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EFFECT OF METAL IONS ON THE CYTOSOLIC NADP$^+$-DEPENDENT ISOCITRATE DEHYDROGENASE FROM GERMINATING BLACKGRAM, *VIGNA MUNGO* (L.) HEPPER

M. Ayub Ali*, W. Joykumar Singh, L. Inaotombi Devi, S. Kunjeshwori Devi and L. Rupachandra Singh

Department of Biochemistry,
Manipur University, Canchipur - 795 003, Manipur

ABSTRACT

The cytosolic NADP$^+$-dependent isocitrate dehydrogenase from germinating blackgram shows an absolute requirement of a divalent metal ion such as Mn^{2+} or Mg^{2+} for expression of its full activity. Other metal ions like Co^{2+}, Zn^{2+} and Cu^{2+} could also activate the enzyme, though the degree of activation was lower than that of either Mn^{2+} or Mg^{2+} at the same concentration. In contrast, three metal ions Sn^{2+}, Ba^{2+} and Ca^{2+} were found to be inhibitory. The kinetics analysis with Mn^{2+} shows sigmoidal response suggestive of a positive co-operativity in metal ion binding. The $A_{0.5}$ was 3.3 µM as deduced from a Hill plot with a Hill coefficient value of 2.1.

INTRODUCTION

The enzyme NADP$^+$- dependent isocitrate dehydrogenase [*threo*-D_s-isocitrate : NADP$^+$ oxidoreductase (decarboxylating), EC 1.1.1.42] (NADP$^+$-IDH) is found to occur in both prokaryotes and eukaryotes. The enzyme catalyses the oxidative decarboxylation of isocitrate to yield a-ketoglutarate with concomitant reduction of NADP$^+$ to NADPH and requires metal ion (Mn^{2+} or Mg^{2+}) for catalysis. The enzyme is more active in the presence of Mn^{2+} than in presence of Mg^{2+} which seems to be a common feature for all NADP$^+$-IDHs studied so far, independent of their origin (Curry and Ting, 1976; Kaur *et al*., 1996). NADP$^+$-IDH is mainly cytosolic enzyme (Henson *et al*., 1986), with only small proportion of the enzyme normally residing in chloroplast (Randall and Givan, 1981), mitochondria (Curry and Ting, 1976) and peroxisomes (Randall and Givan, 1981). In higher plants NADP$^+$-IDH has been purified and characterized from some plant tissues such as green pea leaves and roots (Chen *et al*., 1988), castor bean seeds (Satoh, 1972), maize seeds (Curry and Ting, 1976), pigeonpea root nodules (Kaur *et al*., 1996) and germinating pea seeds (Srivastava and Singh, 2001).

The present paper describes the effect of some divalent metal ions on the cytosolic NADP$^+$-IDH from germinating seeds of blackgram, *Vigna mungo* (L.) Hepper *cv*. yellowish-green seed coat as a prerequisite in making an attempt to elucidate the physiological and metabolic functions of the metal ions on the enzyme.

MATERIAL AND METHODS

Blackgram seed: The seeds of blackgram, *Vigna mungo* (L.) Hepper *cv*. yellowish-green were purchased from local market.

Chemicals: DL- Isocitric acid, NADP$^+$ (sodium salt), DEAE-cellulose, Sephadex G-200, and blue dextran were purchased from Sigma Chemical Company, St. Louis, MO, USA., Tris (hydroxymethyl) aminomethane (Tris) was purchased from Hi Media Laboratories Pvt. Ltd., Bombay; citric acid and 2-mercaptoethanol were from Loba Chemie, Bombay; Ammonium sulphate, polyvinyl pyrrolidone, magnesium chloride, manganese

* Present address: Department of Veterinary Biochemistry, College of Veterinary Sciences and A.H., CAU, Selesih, Aizawl - 796 014 (MIZORAM).

sulphate and copper sulphate from Central Drug House, New Delhi; Blue Sepharose CL-6B was from Pharmacia Fine Chemicals, Uppsala, Sweden. All other reagents were of analytical grade.

Enzyme assay and protein determination: The activity of $NADP^+$-IDH was measured spectrophotometrically by monitoring the rate of formation of NADPH (e_{NADH} at 340 nm = 6.22 x 10^3 M^{-1} cm^{-1}) adopting the procedure of Curry and Ting (1976), in a reaction mixture (0.8 mL) containing appropriately diluted enzyme, 2.0 mM DL-isocitric acid (equivalent to 1.0 mM *threo*-D_s-isocitric acid), 1.0 mM $NADP^+$ and 1.0 mM $MnSO_4$ in 50 mM Tris-HCl buffer pH 7.4 at 30°C. The initial rate of reaction was determined graphically. One enzyme unit was defined as that amount of enzyme which brings about the formation of 1µ mole of NADPH per minute under the assay conditions. The total protein was determined by the method of Lowry *et al.* (1951) using bovine serum albumin as the standard.

Enzyme extraction and purification: The cytosolic $NADP^+$-IDH was isolated and purified from 48 hrs germinated blackgram, *Vigna mungo* (L.) Hepper *cv.* yellowish green seed coat upto electrophoretic homogeneity by successive purification steps including homogenization, heat treatment (55°C), ammonium sulphate fractionation (50-80% saturation), ion-exchange chromatography on DEAE-cellulose and affinity chromatography on Blue Sepharose CL-6B. The purified enzyme was found to have a specific activity of 214.6 units/mg protein which is 429-fold higher than that of the initial crude enzyme preparation.

RESULTS AND DISCUSSION

The purified cytosolic $NADP^+$-dependent isocitrate dehydrogenase ($NADP^+$-IDH) from blackgram exhibited a requirement for divalent metal ion such as Mn^{2+} or Mg^{2+} for expression of its full activity. This is in agreement with the reports concerning several forms of $NADP^+$-IDH from various sources (Kaur *et al.*, 1996; Murakami *et al.*, 1997). As reported earlier for the enzyme from various sources (Chen *et al.*, 1988; Kaur *et al.*, 1996), Mn^{2+} was found to be more effective in activating the enzyme than Mg^{2+}. At equimolar concentrations (1.0 mM), Mg^{2+} was found to be about 33% as effective as Mn^{2+} in activating purified cytosolic $NADP^+$-IDH in an otherwise standard assay mixture. Similar results were also reported for the enzymes from maturing castor bean (Satoh, 1972), pea (Randall and Givan, 1981; Chen *et al.*, 1988) and root nodule of pigeonpea (Kaur *et al.*, 1996). Besides Mn^{2+} and Mg^{2+}, other metal ions, *viz.*, Co^{2+}, Zn^{2+} and Cu^{2+} could also activate the cytosolic $NADP^+$-IDH though the degree of activation was lower than that of either Mn^{2+} or Mg^{2+} at the same concentration. These results were found to be similar to those of the enzymes from maturing castor bean (Satoh, 1972), germinating pea seeds (Srivastava and Singh, 2001) and an animal source (Kratochvil *et al.*, 1967), except that Cu^{2+} was inhibitory in the case of the castor bean enzyme (Satoh, 1972). In contrast to these activating divalent metal ions, the three metal ions - Sn^{2+}, Ba^{2+} and Ca^{2+} were found to be inhibitory. The inhibitory effect of Sn^{2+} (67% inhibition) was highest among the three inhibitory divalent metal ions and that of Ba^{2+} (34% inhibition) was, in turn, higher than that of Ca^{2+} (17% inhibition). These experimental observations are more clearly brought out in Table 1 in which the enzyme activities in presence of different divalent metal ions are compared.

To study the effect of concentration of Mn^{2+} on the activities of the cytosolic $NADP^+$-IDH, the initial rate of the enzymatic reaction was determined with increasing concentrations of Mn^{2+} in an otherwise standard reaction mixture. In the absence of

Table 1. Effect of some divalent metal ions on the activities of cytosolic NADP$^+$-IDH from germinating blackgram, *Vigna mungo* (L.) Hepper, *cv.* yellowish green seed coat

Divalent cation (1 mM)	Enzyme activity (%)*
No metal	100
Mn^{2+}	370
Mg^{2+}	190
Co^{2+}	166
Zn^{2+}	150
Cu^{2+}	133
Ca^{2+}	83
Ba^{2+}	66
Sn^{2+}	33

The enzyme activity was determined spectrophotometrically by monitoring the formation of NADPH at 340 nm (ε_{NADPH} = 6.22 x 10^3 M^{-1} cm^{-1}) in presence of each of the different divalent cations at 1.0 mM in a reaction mixture (0.8 mL) containing 2.0 mM DL-isocitric acid and 1.0 mM NADP$^+$ in 50 mM Tris-HCl buffer pH 7.4 at 30°C. The reaction was started with the addition of constant amount of the enzyme. The data are expressed as percent enzyme activity of that without externally added metal ion in the test mixture.

any externally added metal ion, the purified cytosolic NADP$^+$-IDH was found to exhibit activities which are about 27% of the corresponding maximal activities observed in the standard assay system with Mn^{2+} as the cofactor. These results are in agreement with those of earlier reports for the purified NADP$^+$-IDHs from some sources including maturing castor bean seeds (Satoh, 1972) and pea leaf chloroplast (Randall and Givan, 1981). These baseline activities expressed in the absence of any externally added metal ion is probably due to some catalytic metal ions endogenously bound to the enzyme proteins. The remaining 73% of the full activity was expressed in a concentration-dependent manner as the concentration of Mn^{2+} in the reaction mixture was gradually increased. The saturation curve with respect to the expression of the externally added metal ion dependent activity was found to be sigmoidal in shape suggestive of a positive cooperativity in the metal ion binding (Fig. 1). The $A_{0.5}$ value for Mn^{2+}, the concentration of the metal ion required for the expression of 50% of the externally added Mn^{2+}-dependent activity, was 3.3 µM as deduced from a Hill plot with a Hill coefficient value of 2.1 (data not shown). Comparable results were also reported earlier for NADP$^+$-IDHs from other sources (Satoh and Nakamura, 1984; Kaur *et al.*, 1996; Murakami *et al.*, 1997). The saturation curves of pig heart enzyme with respect to the substrate *threo*-D_s-isocitrate complexed with the metal ions including Mn^{2+}, Cd^{2+}, Co^{2+} and Zn^{2+} ions were found to be sigmoidal, indicating the allosteric nature of the dehydrogenase. The Hill interaction coefficients of *threo*-D_s-isocitrate complexed with Mn^{2+}, Cd^{2+}, Co^{2+} and Zn^{2+} were 1.90, 1.75, 1.28 and 1.12, respectively. Saturation kinetics of the substrate-metal complexes including Mg^{2+}, Fe^{2+} and Ni^{2+} ions were, however, found to exhibit normal hyperbolic curves with Hill coefficients of 1. The ionic radii of metal cations were found to be closely correlated with the maximal velocity, the enzyme affinity and the Hill coefficient values for the substrate-metal complexes (Murakami *et al.*, 1997). Similar case of allosteric behaviour in a higher plant NADP$^+$-IDH was also reported by Kaur *et al.* (1996). Unlike the enzyme from most other plant tissues (Chen *et al.*, 1988), Mn^{2+} evoked a sigmoidal response with the enzyme from pigeonpea root nodule. The apparent $A_{0.5}$ for Mn^{2+} was 0.3 mM (Kaur *et al.*, 1996). Satoh and Nakamura (1984) performed kinetic studies on the metal ion dependence of the enzyme activity with

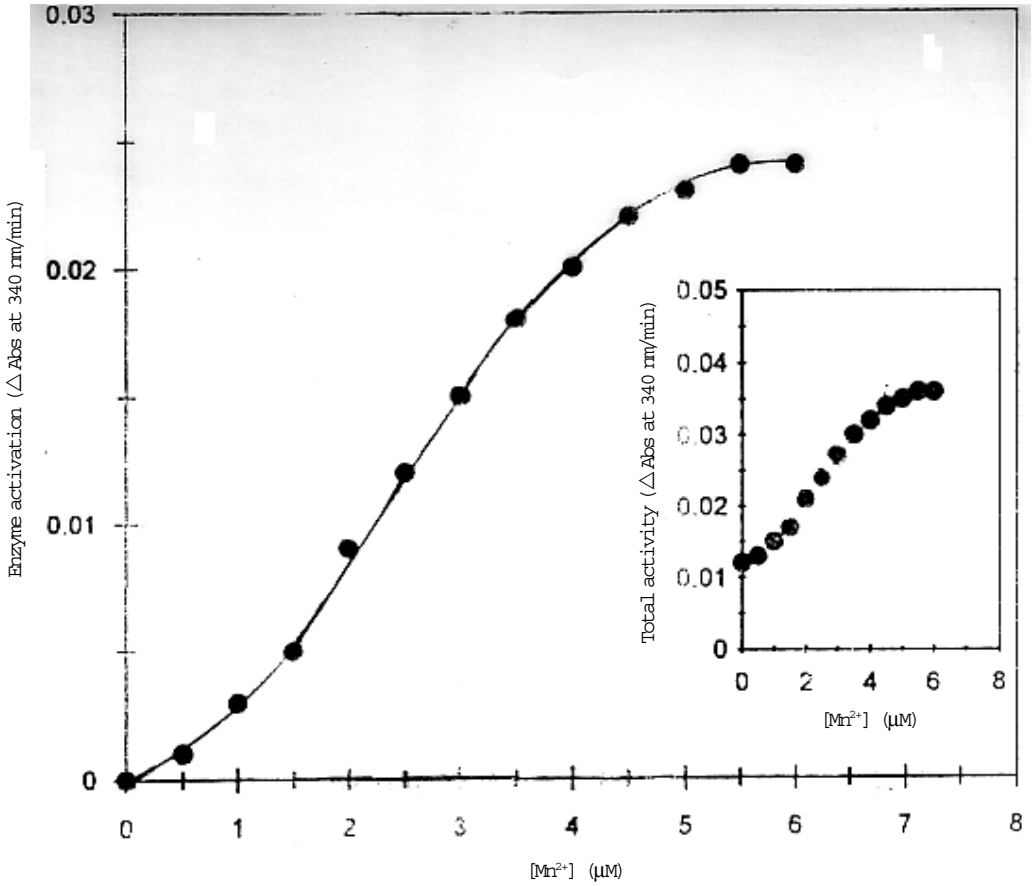

Fig.1. Effect of Mn^{2+} concentration on the externally added metal ion-dependent activity of cytosolic $NADP^+$-IDH in 50 mM Tris-HCl buffer pH 7.4 at 30°C at 1.0 mM *threo*-D_s-isocitrate and 1.0 mM $NADP^+$. Reaction was started with the enzyme (27 ng/mL in test) and monitored spectrophotometrically at 340 nm. The inset shows the plot of the total enzyme activity (baseline activity plus the externally added Mn^{2+}-dependent activity) against Mn^{2+} concentration.

respect to the reverse reaction (reductive carboxylation of α-ketoglutarate) catalysed by the purified castor bean soluble $NADP^+$-IDH. The activation curve obtained with metal ion (Mg^{2+}, Mn^{2+}, Zn^{2+} or Co^{2+}) was found to be sigmoidal in shape, indicating allosteric nature of the enzyme. The $A_{0.5}$ value for Mg^{2+} was 12 μM as inferred from a Hill plot with a Hill coefficient value of 1.3.

The possible roles of divalent metal ions like Mn^{2+} or Mg^{2+} in the reaction catalysed by $NADP^+$-IDH may include (i) the metal ion may complex with the substrates, D-isocitrate and α-ketoglutarate, to enable them to be properly bound to the enzyme for the reaction to proceed, (ii) the metal ion may also have some important roles in the catalysis process itself and (iii) the metal ion may have regulatory role in the instances where the enzyme gives sigmoidal response in the dependence of enzyme activity on metal ion concentration. The results of the present study on the effect

of metal ions on the $NADP^+$-IDH from blackgram lends support to these ideas regarding the roles of the divalent metal ions in the reaction catalysed by the enzyme. As the baseline activity observed for cytosolic $NADP^+$-IDH was 27% of the maximal activity observed in the standard assay system with Mn^{2+} as the cofactor, it is presume that some of the binding sites in the enzyme might be already saturated with the endogenously bound metal ion.

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