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Role of Metabolites and Significance of SH Groups in the Action of NADP$^+$-Linked Isocitrate Dehydrogenase of Urdbean Seeds (*Phaseolus mungo* L.)

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ABSTRACT

NADP$^+$-linked-isocitrate dehydrogenase (EC 1.1.1.42) is a key enzyme of the Tricarboxylic Acid Cycle (TCA) and has been purified from urdbean seeds and it is inhibited by ATP in a competitive manner having inhibitor constant (K_i) 1.32 mM. Phosphoenol-pyruvate, an energy rich compound plays an important role in the regulation of this enzyme and this metabolite inhibited the enzyme activity of NADP$^+$-linked-isocitrate dehydrogenase of urdbean with inhibitor constant (K_i) 2.66 mM in a competitive manner. The mode of inhibition by various metabolites of Krebs cycle has been carried out and found that oxaloacetate and succinate inhibit ICDH urdbean enzyme in a competitive manner with respect to isocitrate and their K_i values are found to be 7.27 and 10.67 mM, respectively. Citrate inhibits the urdbean ICDH enzyme non competitively with K_i value equal to 3.33 mM. The SH groups play a important role in the activity of NADP$^+$-linked-isocitrate dehydrogenase and blocking of this group with SH-reagents, leads to inactivation of urdbean ICDH enzyme. With excess iodoacetamide (1.00 mM) and N-ethylmaleimide (4.0 mM) inhibition of this enzyme follows first order kinetics, suggesting that there are four reactive SH groups per mole of enzyme which are equally reactive and there is no site- site interaction among the tetrameric isoicitrate dehydrogenase of urdbean.

Key words: ATP, phosphoenolpyruvate, Krebs cycle, SH group, enzyme kinetics, non-competitive inhibition

INTRODUCTION

Dehydrogenases are important respiratory enzymes in all organisms. Microbial dehydrogenases control biogeochemical cycles and are considered as determining factor for soil quality and its fertility (Subhani *et al*., 2001; Sajjad *et al*., 2002; Matinizadeh *et al*., 2008).

Isocitrate dehydrogenase (ICDH; EC 1.1.1.42) catalyzes the reversible oxidative decarboxylation of isocitrate to α-ketoglutarate and with NAD$^+$ or NADP$^+$ as cofactor. Isocitrate dehydrogenase occurs in a wide range of species in all domains of life, including the organisms that lack complete citric acid cycle (Wang and Lau, 1996). Both NAD$^+$ and NADP$^+$-dependent isoforms of ICDH exist in plants (Hodges *et al*., 2003). The NAD$^+$-linked (ICDH) restricted to mitochondria is a part of the tricarboxylic acid (TCA) cycle, while NADP$^+$-linked (ICDH) are typically found in

the cytosol (Srivastava *et al.*, 2001) with small portion of activity also in chloroplasts and peroxisomes, as well as in mitochondria that exhibit normal hyperbolic activities. The cytosolic ICDH is responsible for up to 90% of the $NADP^+$-dependent activity in leaf extracts (Hodges, 2002). Both isoforms are also associated with nitrogen signal or its status (Dutilleul *et al.*, 2005) and also associated in ammonia assimilation (Lemaitre *et al.*, 2007). It also promotes redox signalling or homeostasis in response to oxidative stress by NADPH (Hodges *et al.*, 2003). In yeast and in animals, there is some evidence that cytosolic ICDH plays such a role (Yang and Park, 2003). Its molecular weight from various sources and its structure are reported to be dimeric protein. However, our enzyme isolated from urdbean is tetrameric protein made up of four identical monomers (Srivastava *et al.*, 2010). Popova *et al.* (1986) reported the inhibition by ADP and ATP in competitive manner for ICDH in pea leaves. Several metabolites of Krebs cycle play significant role in the regulation of enzyme activity as it is evident from inhibition pattern discussed by different workers (Marr and Weber, 1969; Popova, 1993).

The significance of SH group in the activity of isocitrate dehydrogenase has been reported in castor bean seeds by p-choloromercuric benzoate (Satoh, 1972). It is also reported that the inactivation of ICDH in *Azotobacter vinelandii* by irradiation technique with radical anions $(CNS)^-$ and Br^- generated in gamma irradiation solution at pH 6.5. This may be due to oxidation of sulfhydral groups which are present on the active site of the enzyme (Schubert *et al.*, 1979). In case of neurodegenerative diseases, selective dehydrogenase like ICDH is studied well in order to observe their altered expression and catalytic activity (Sushma *et al.*, 2007). The present study was undertaken to investigate how the metabolites of TCA and energy rich compound such as ATP, ADP and PEP are associated with regulation of ICDH.

MATERIALS AND METHODS

$NADP^+$-isocitrate dehydrogenase of urdbean seed was isolated as described earlier (Srivastava *et al.*, 2010).

$NaH_2PO_4.2H_2O$, $Na_2HPO_4.2H_2O$, Tris (hydroxyl methyl) aminomethane and DL-isocitric acid of analytical grade were purchased from Sisco Research Laboratory (SRL) Bombay, iodoacetamide iodoacetate, N-ethylmaleimide and cysteine were purchased from Sigma Chemical Company, U.S.A., 5,5' dithiobis-(2 nitrobenzoic acid) from S.R.L. Bombay. Methylene blue was from Qualigens Fine Chemicals, ATP, ADP, Nicotinamide adenine dinucleotide phosphate were purchased from S.R.L. Bombay. AMP, oxaloacetic acid, α-ketoglutarate citric acid and succinic acid were from Sigma Chemicals Company St.Louis, USA. All solutions were prepared in double distilled water from an all glass (borosil) assembly.

Native PAGE 10% at pH 7, 7.5 and 8.5 revealed single protein band with amidoblack staining suggesting that enzyme preparation is to be homogeneous SDS-PAGE also gave a single band suggesting that ICDH is made up of identical subunits (Srivastava *et al.*, 2010).

The enzyme activity has been measured by monitoring the rate of formation of ICDH at 366 nm which is produced as a result of oxidation of isocitrate and aliquot (0.79 mL) of 50 mM phosphate buffer pH 7.5 containing isocitrate (2.5 mM), $NADP^+$ (0.62 mM) and $MgCl_2$ (3.75 mM) was brought to 30°C. The reaction was started by adding 0.01 mL of suitably diluted enzyme and the rate of increase in absorbance was noted at 50 sec intervals at 366 nm. The enzyme activity was calculated from ϵ_{NADPH} value (3.11×10^3 M^{-1} cm^{-1}). The unit of enzyme activity was defined as amount of enzyme which brings about the formation of 1 μmole of NADPH in one minute under the test condition as defined above.

The enzyme and desired reagents (ATP, ADP, AMP, oxaloacetate, succinate, α-ketoglutarate, citric acid and phosphoenolpyruvate) were incubated at specified concentration in 50 mM phosphate buffer pH 7.5 at 30°C. Aliquots were withdrawn at specified time intervals and were assayed for enzyme activity with or without addition of $MgCl_2$ in the test mixture. The total number of SH groups of native ICDH from urdbean were estimated by studying its reaction with 5, 5' dithiobis-(2-nitrobenzoate). Appearance of yellow coloured product 2-nitromercaptobenzoate was monitored at 405 nm (Ellman, 1959). The ϵ_{405} was determined by titration of freshly prepared cysteine solution with DTNB and found to be 1.14×10^4 M^{-1} cm^{-1}.

RESULTS

Effect of nucleotides: $NADP^+$-linked-isocitrate dehydrogenase of urdbean was inhibited by ATP and ADP while AMP did not illustrated significant change in the activity of urdbean enzyme. At low concentration (4.0 mM) both ATP and ADP revealed little inhibition but at higher concentration of each compound, the inhibition of urdbean enzyme was enhanced. The order of inhibition of $NADP^+$-linked ICDH enzyme by nucleotides in the following manner ATP≥ADP>AMP (Table 1). The kinetics of inhibition of urdbean enzyme illustrated that ATP inhibits the enzyme in competitive manner with respect to substrate(isocitrate) (Fig. 1) and the value of inhibitor constant (K_i) of ATP for this enzyme was found to be 1.32 mM.

Effect of phosphoenol pyruvate: Phosphoenol pyruvate is an energy rich compound, which participates both in the conversion of seed fat depot into carbohydrates and the breakdown of the later for energy generation. It was found that this metabolite inhibits the urdbean enzyme competitively with respect to substrate. The kinetic pattern of inhibition of phosphoenol pyruvate for $NADP^+$-linked-isocitrate dehydrogenase (ICDH) of urdbean was determined and found to be 2.66 mM (Fig. 2). Hence the competitive nature of inhibition of purified enzyme (ICDH) by phosphoenol pyruvate suggested that this metabolite involved in the regulation of isocitrate dehydrogenase.

Effect of metabolites of Krebs cycle: The role of different metabolites of Krebs cycle such as citrate, succinate, oxaloacetate and α-ketoglutarate were carried out on the mode of action of urdbean ICDH enzyme (Table 2). All the metabolites of Krebs cycle were found to be inhibitory for this enzyme. The mode of action of these metabolites was confirmed by studying the kinetic of inhibition of urdbean ICDH enzyme in presence of above metabolites (Fig. 3 and 4). From the plots,

Table 1: Effect of different nucleotides on the activity of $NADP^+$-linked isocitrate dehydrogenase urdbean

Nucleotides	Concentration (mM)	%Residual activity	%Inhibition
Control	0	100	0
ATP	4	80	20
	8	50	50
ADP	4	85	15
	8	66	34
AMP	4	100	00
	8	100	00

Enzyme solution (0.04 mg mL^{-1}) and nucleotides in 50 mM phosphate buffer, pH 7.5 were incubated at 30°C for 5 min before starting the reaction with the substrate, co-enzyme and metal ions. The enzyme activity was monitored by the rate of change of absorbance at 366 nm

Table 2: Effect of metabolites of Krebs cycle on the activity of urdbean isocitrate dehydrogense

Metabolites	Concentration (mM)	%Residual activity	%Inhibition
Oxaloacetic acid	2	80	20
	4	65	35
	8	50	50
α-ketoglutarate	4	85	15
	8	45	55
Succinate	2	75	25
	4	65	35
	8	48	52
Citric acid	4	70	30
	8	47	53

Enzyme solution (60 μg mL^{-1}) and metabolites in 50 mM phosphate buffer, pH 7.5 were incubated at 30°C for 5 min before starting the reaction with the substrate (2.5 mM)

Table 3: Inhibitor constants of metabolites of Krebs cycle, for urdbean isocitrate dehydrogenase enzyme

Metabolites	Type of inhibition	Inhibitor constant (K_i) mM
Oxaloacetic acid	Competitive	7.27
α-ketoglutarate	Competitive	6.67
Succinate	Competitive	10.67
Citric acid	Non-competitive	1.33

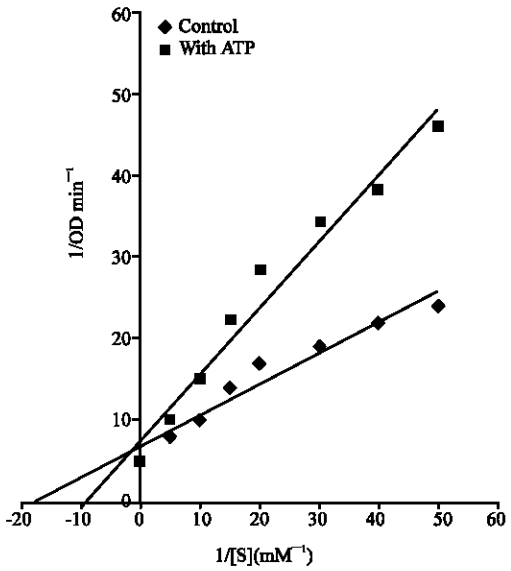

Fig. 1: Double reciprocal plots for the effect of DL-isocitrate concentration on urdbean ICDH activity in the absence (control) and in the presence of 4.0 mM ATP .The activity was estimated as described in assay procedure.The enzyme concentration was 5.0 μ g mL^{-1}. Reaction rate is expressed in terms of change in OD at 366 nm

it is clear that α-ketoglutarate, succinate and oxaloacetate inhibited the urdbean ICDH enzyme in a competitive manner whereas inhibition with citric acid exhibited non-competitive pattern with respect to substrate. The values of inhibitor consant (K_i) with different TCA cycle metabolites for urdbean enzyme as shown in Table 3.

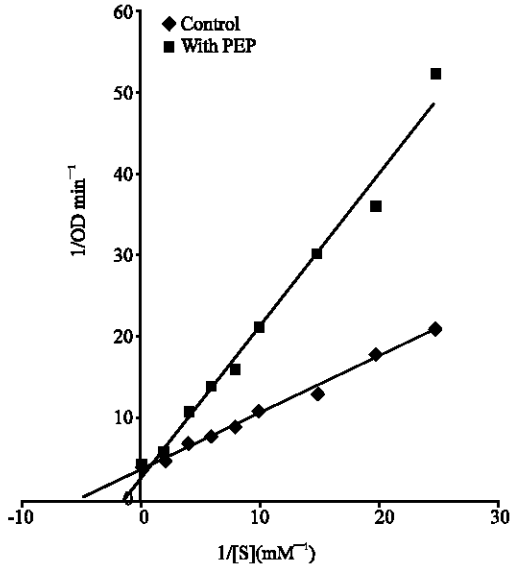

Fig. 2: Double reciprocal plots for the effect of DL-isocitrate concentration on urdbean ICDH activity in the absence (control) and in the presence of 4.0 mM phosphoenolpyruvate (PEP). The activity was estimated as described in assay procedure. The enzyme concentration was 5.0 μ g mL^{-1}. Reaction rate is expressed in terms of change in OD at 366 nm

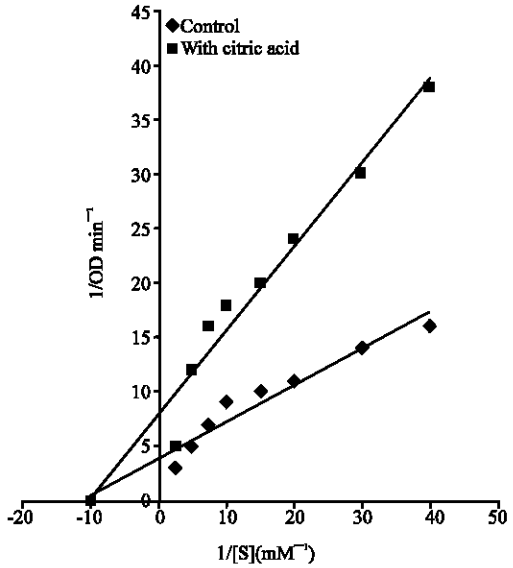

Fig. 3: Double reciprocal plots for the effect of DL-isocitrate concentration on urdbean ICDH activity in the absence (control) and in the presence of 4.0 mM citric acid. The activity was estimated as described in assay procedure. The enzyme concentration was 5.0 μ g mL^{-1}. Reaction rate is expressed in terms of change in OD at 366 nm

Estimation and significance of SH groups for enzyme activity: The SH group of urdbean isocitrate dehydrogenase was estimated by its interaction with 5-5-dithiobis (2-nitrobenzoate) i.e.,

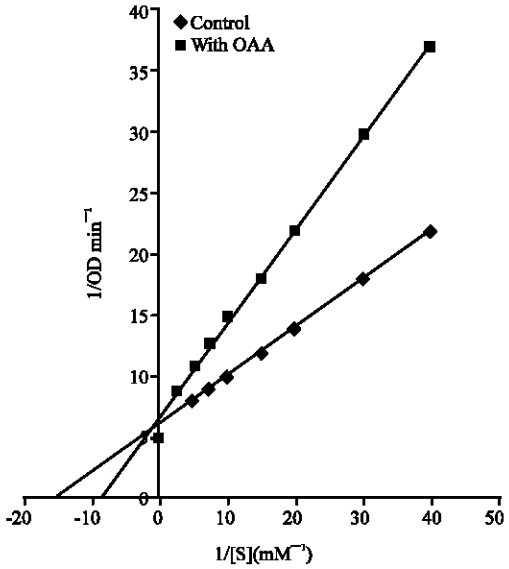

Fig. 4: Double reciprocal plots for the effect of DL-isocitrate concentration on urdbean ICDH activity in the absence (control) and in the presence of 4.0 mM oxaloacetic acid. The activity was estimated as described in assay procedure. The enzyme concentration was 5.0 μ g mL^{-1}. Reaction rate is expressed in terms of change in OD at 366 nm

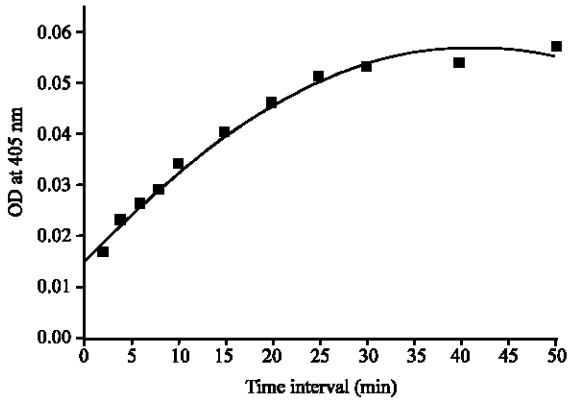

Fig. 5: Estimations of reactive SH groups in native $NADP^+$-linked ICDH of urdbean in interaction with DTNB. To a solution of DTNB in 50 mM phosphate buffer pH 7.5 (0.75 mL) was added to 0.05 mL of enzyme. OD was noted at different time intervals. Final concentration of DTNB and enzyme was 0.32 and 0.20 mg mL^{-1}, respectively

DTNB and time dependent increase in absorbance at 405 nm as a result of adding ICDH enzyme with excess DTNB as illustrated in (Fig. 5) and it was appeared that this enzyme has some fast reacting SH groups. The total numbers of fast reacting SH-groups were found to be 4.0 per mole of ICDH and thus one reactive SH groups per monomeric 32, 000-33, 000 of urdbean enzyme. To confirm the significance of SH groups, ICDH enzyme was inactivated on treatment with SH reagents i.e., iodoacetamide, N-Ethyl-Maleimide (NEM) and p-chloromercuric benzoate. It was observed that ICDH enzyme lost their activity in single exponential manner in presence of

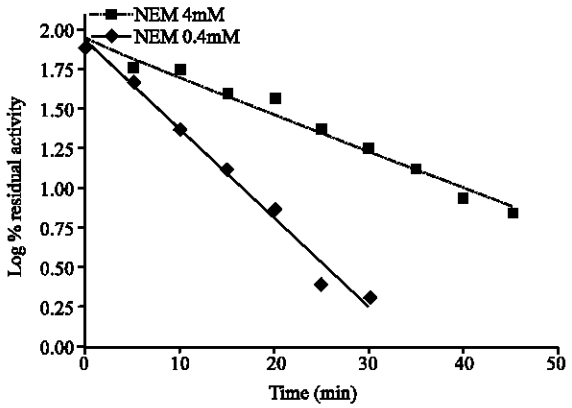

Fig. 6: Kinetics of inactivation of urdbean NADP$^+$-linked ICDH with N-ethylmalemide. The solution of enzyme (0.04 mg mL^{-1}) and NEM (0.4 and 4.0 mM) were incubated in 50 mM phosphate buffer pH 7.5 at 30°C. The aliquots were withdrawn at different intervals of time and assayed for activity as usual

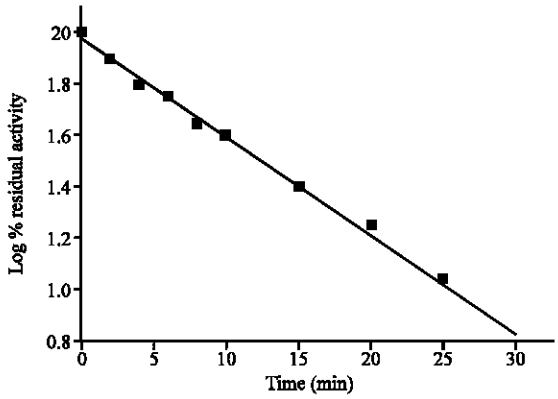

Fig. 7: Kinetics of inactivation of urdbean NADP$^+$-linked ICDH with iodoacetamide. The enzyme solution (0.02 mg mL^{-1}) and iodoacetamide (1.0 mM) were incubated in 50 mM phosphate buffer (pH 7.5) at 30°C. The aliquots were withdrawn at different intervals of time and assayed for activity as usual

iodoacetamide and NEM. Hence in each case, simple first order kinetics were observed as would be expected when one of the reactant is present in large molar excess i.e., 4.0 mM NEM (Fig. 6) and with 1.0 mM iodoacetamide (Fig. 7). The pseudo first order rate constant at 1.0 mM iodoacetamide was found to 0.092 whereas at 0.4 and 4.0 mM were found to be 0.099 and 0.173 min^{-1}, respectively. The loss of urdbean ICDH enzyme activity in presence of SH-groups revealed that the some of the SH-groups are concerned with catalytic action of this enzyme. Furthermore, simple first order kinetics in these cases revealed that there was no site-site interaction within the tetrameric ICDH protein as for as reaction with SH-reagents are concerned.

DISCUSSION

The activity of urdbean ICDH is strongly influenced by nucleotides suggesting that regulatory role of this enzyme. The order of effect of ATP, ADP and AMP on urdbean enzyme are,

ATP≥ADP>AMP. The ATP inhibits the ICDH in competitive manner with K_i equal to 1.32 mM. Earlier studies also reported that the kinetics of inhibition of ICDH by ATP was found to be mixed type with respect to $NADP^+$ and isocitrate and their K_i values were 1.12×10^{-3} and 1.10×10^{-3} M, respectively in *Rhodospirillum rubrum* (Dhilion and Marvin, 1972). The nature of inhibition may be probably due to removal of bound metal ions by ATP at the active site of enzyme. Similar type of inhibition was reported with ATP and ADP from enzyme of pea leaves (Popova *et al*., 1986). ATP had little inhibitory effect on enzyme from *Paecilomyces varioli* (Takao *et al*., 1986). In present study the energy rich compound phosphoenol puruvate also inhibits the activity of urdbean ICDH in a competitive manner with K_i value equal to 2.66 mM. Similar type of inhibition was also reported by $NADP^+$ linked isocitrate dehydrogenase enzyme of alkalophilic *Bacillus* in compounds like glyceraldehydes 3-phosphate, 3-phosphoglycerate and phosphoenol pyruvate (Shikata *et al*., 1988).

Several metabolites of Krebs cycle have been tested for inhibitory action. It was observed that citrate inhibited the enzyme non-competitively with K_i value equals to 1.33. The enzyme from *A. niger* (Mattey and Bowes, 1979) was inhibited by citrate but was not affected by malate. The inhibition of enzyme activity by citrate appears to be sensitive at pH 7.6 but citrate showed competitive inhibition with respect to isocitrate in pea leaves (Popova *et al*., 1986). We have observed that oxaloacetate, succinate and α-ketoglutarate inhibit the urdbean enzyme in a competitive manner with K_i value equal to 7.27, 10.67, 6.67 mM, respectively. The partially purified $NADP^+$-ICDH from *Brevibacterium flavum* (Shiio and Ozaki, 1968) and also from *Bacillus, E.coli* and pig heart was strongly inhibited by oxaloacetatate in a competive inhibition with respect to isocitrate. The α-ketoglutarate with respect to isocitrate inhibited the enzyme ICDH from *Pacilomyces variole* competitively (Takao *et al*., 1986). The effect of TCA cycle intermediate at 4.0 mM of oxaloacetate and succinate showed 35% inhibition of ICDH while at same concentration α-ketoglutarate illustrated little inhibition about 15%. Even at lower concentration (2.0 mM) both oxaloacetate and succinate illustrated 20 and 30% enzyme inhibition respectively. But at higher concentration (8.0 mM), all TCA cycle intermediate illustrated near about 50% inhibition of ICDH enzyme. Earlier reports have also been confirmed our finding of inhibition of urdbean ICDH by Krebs cycle intermediates in various organisms (Mattey and Bowes, 1979; Shiio and Ozaki, 1968; Takao *et al*., 1986).

The significance of SH groups in the activity of urdbean $NADP^+$-linked isocitrate dehydrogenase was observed. $NADP^+$-linked isocitrate urdbean one reactive SH group per monomeric subunit (molecular weight 32, 000-33, 000) which can be titrated with 5-5' dithiobis-(2-nitro benzoate). The denatured urdbean ICDH possesses total of 8.0 SH-groups per mole, i.e., each monomer have 2 SH groups in which one is more reactive than the other. Blocking of this group with SH-reagents, leads to inactivation of urdbean ICDH enzyme. With excess iodoacetamide (1.00 mM) and N-ethylmaleimide (4.0 mM) the inhibition of urdbean enzyme obeys first order kinetics and the rate constants were found to be 0.092 and 0.173 min^{-1}, respectively and these results suggesting that the four SH groups are equally reactive and there is no site-site interaction among the tetrameric isocitrate dehydrogenase of urdbean. Earlier study have been reported similar type of finding with blocking SH groups by 0.01 M iodoacetate at pH 5.0 and obeyed pseudo first order kinetics indicating that a methionine residue is a critical for the function of the human heart enzyme (Seelig and Colman, 1979). One of the study has also been confirmed the significance of SH groups in the activity of $NADP^+$-linked isocitrate dehydrogenase which are present on the active site of enzyme in the *Azotobacter vinelandii* (Schubert *et al*., 1979). Thus the

overall finding of present studies with urdbean ICDH showed inhibition with the metabolites of Krebs cycle, ATP and energy rich compound phosphoenolpyruvate. It was observed that ATP, phosphoenolpyruvate, oxaloacetate, α-ketoglutarate and succinate have competitive inhibition with respect to isocitrate while citric acid shown non competitive inhibition with urdbean ICDH. The significance and estimation of SH group in ICDH of urdbean have been determined and found that one reactive SH group per monomeric subunit and denatured ICDH possesses 8.0 SH-groups per mole of ICDH of urdbean. Thus this result concludes that the purified $NADP^+$-linked isocitrate dehydrogenase from urdbean, is a regular tetramer and all the monomers are equally reactive.

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