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Regulation of NAD$^+$- Specific Isocitrate Dehydrogenase from *Pythium ultimum*

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The NAD$^+$-specific activity of a dual coenzyme-specific isocitrate dehydrogenase (IDH; EC 1.1.1.41) from the primitive fungus *Pythium ultimum* was investigated to elucidate the regulatory factors that may influence the intracellular distribution of carbon and the availability of intermediates, e.g. citrate, for fatty acid synthesis. Inhibition of NAD$^+$-IDH activity by diphospho- and triphosphonucleotides (ATP, ADP, and GTP) reflected the sensitivity of this enzyme to cellular energy charge even though monophosphonucleotides (AMP and GMP) had little effect on activity. NADPH, but not NADH, substantially inhibited NAD$^+$-IDH activity, showing noncompetitive inhibition with isocitrate. Oxalacetate and α-ketoglutarate showed competitive inhibition with isocitrate, while citrate and *cis*-aconitate showed mixed-noncompetitive inhibition with isocitrate. Inhibition by these substances ranged from 29 to 46% at 10 mM. The inhibitory effect of oxalacetate was increased synergistically by glyoxylate, which alone caused 31% uncompetitive inhibition at 10 mM, and a mixture of the two substances at 1 mM each showed 98% inhibition of NAD$^+$-IDH activity. The regulation of NAD$^+$-IDH in *Pythium ultimum* seems to be a complex process involving mitochondrial metabolites. The addition of glyoxylate (3 mM) and oxalacetate (3 mM) to the culture medium resulted in the production of 49% more lipid by *P. ultimum*.

Keywords: Fungi, Isocitrate dehydrogenase, Lipid, Regulation.

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Introduction

NAD$^+$-dependent isocitrate dehydrogenase (D-*threo*-isocitrate:NAD$^+$ oxidoreductase, EC 1.1.1.41; NAD$^+$-IDH) is a widely studied enzyme that catalyzes the oxidative decarboxylation of isocitrate to α-ketoglutarate. It is a key regulatory enzyme for the control of carbon flow through the TCA cycle. Control of this enzyme is complex and, although there appear to be regulatory features in common, detailed modes of regulation of this enzyme may vary from plant, animal, and microbial sources. Common regulatory features of NAD$^+$-IDH are activation by Mg^{+2}, modulation by citrate as a positive allosteric effector, and inhibition by NADH and NADPH.

NAD$^+$-IDH from *Saccharomyces cerevisiae* is one of the most throughly studied examples of this enzyme from yeast or fungal sources. AMP (Kornberg and Pricer, 1951; Hathaway and Atkinson, 1963) is a positive allosteric effector of yeast NAD$^+$-IDH, and citrate acts allosterically to activate or inhibit this enzyme (Gabriel and Plaut, 1991). Similar regulatory properties have been described for this enzyme from other yeast and fungal sources such as *Candida lipolytica* (Mitsushima *et al.*, 1978), *Blastocladiella emersonii* (Le'John *et al.*, 1969), *Neurospora crassa* (Sanwal and Stachow, 1965; Nealon and Cook, 1979), and *Rhodosporidium toruloides* CBS 14 (Evans and Ratledge, 1985). In the latter species, NAD$^+$-IDH showed an absolute specificity for AMP and is believed to be important in regulating the supply of substrate to the key enzyme (ATP:citrate lyase) in the oleaginicity of this organism (Evans *et al.*, 1983). In this high oil (triacylglycerol)-producing yeast, depletion of the nitrogen source from the medium in the later stages of culture development results in reduced intracellular AMP which in turn leads to lower NAD$^+$-IDH activity. Under these conditions, carbon is diverted for oil production via citrate (Botham and Ratledge, 1979).

Pythium ultimum is an oomycetous fungus that produces the polyunsaturated fatty acids arachidonic acid and

eicosapentaenoic acid (Kerwin and Duddles, 1989; Gandhi and Weete, 1991), which have dietary health implications (Simopoulos, 1989). Some strains have the potential to produce relatively high lipid content, e.g. 50% of the dry biomass (Bowman and Mumma, 1967), but the lipid content of the *Pythium* strain used in this study does not exceed 20% and, unlike the oleaginous yeast described above, it does not respond to nitrogen depletion from the medium with regard to oil accumulation (Gandhi and Weete, 1991).

This prompted us to isolate and characterize NAD^+-IDH from *P. ultimum* strain No. 144 (Kim *et al.*, 1996) and determine how certain nucleotides and intermediary metabolites might be involved in the regulation of this enzyme. Previous studies in this laboratory have shown that NAD^+-IDH from *P. ultimum* has several unique properties relative to the enzyme from other fungal sources, e.g. it is a dimer of 96 kDa and is dual-specific for NAD^+ and $NADP^+$ (Kim *et al.*, 1996). In this study, we provide evidence that factors other than AMP and citrate may be more important in the modulation of this enzyme from *P. ultimum*. In particular, glyoxylate/oxalacetate in combination is a powerful inhibitor of NAD^+-IDH.

Materials and Methods

Chemicals Unless otherwise noted, Tris (hydroxy methylamino methane), *threo*-DL-isocitrate, DEAE-Sephacel anion exchange resin, hydrophobic interaction affinity chromatography media (PHE-5), red affinity chromatography media (RR 120-5), Sephadex G-25 and G-100, and other chemicals were from the Sigma Chemical Co. (St. Louis, USA). Dye reagents for the protein assay were purchased from Bio-Rad Laboratories (Richmond, USA).

Fungal source and cultivation *Pythium ultimum* strain No. 144, obtained from D.J.S. Barr of the Biosystematic Research Center, Central Experimental Farm (Ottawa, Canada), was maintained as described previously (Gandhi & Weete, 1991) at 24°C with rotary shaking at 120 rpm. The fungus was transferred to new medium at approximately 6 d intervals. For the purification of proteins, mycelia were collected three days after inoculation by suction filtration in a Buchner funnel and washed with deionized water. The washed mycelia were stored at −20°C prior to use.

Protein extraction Frozen mycelia were thawed in 50 mM potassium phosphate buffer (pH 7.5) containing 2 mM $MgCl_2$, 2 mM β-mercaptoethanol, 1 mM dithiothreitol (DTT) (buffer A) and disrupted with a bead beater from Biospec (Bartleville, USA). Glass beads (0.45–0.5 mm diameter) were added to the thawed mycelia (1:1 dry weight of mycelia). Mycelia were broken by six 30 s beater bursts operation with a 10 s delay between bursts. The mycelial homogenate was centrifuged at 15,000 × *g* for 10 min and the resulting supernatant was centrifuged at 30,000 × *g* for 20 min. The supernatant from the second centrifugation was used as the crude enzyme extract. All enzyme purification steps were carried out at 4°C.

Partial purification of NAD^+-IDH The crude extract was applied directly to a DEAE-Sephacel column (2.5 × 7 cm) that had been washed with 3 vol of buffer B (10 mM potassium phosphate, pH 7.6; 1 mM $MgCl_2$ and 1 mM DTT) and equilibrated at a flow rate of 50 ml h^{-1}. The enzyme was eluted with a stepwise linear gradient of KCl (50–300 mM) and fractions (5 ml) containing NAD^+-IDH activity were collected and combined, and the protein was then precipitated with 80% ammonium sulfate. The precipitate was collected by centrifugation at 20,000 × *g* for 20 min and desalted through a Sephadex G-25 column (2.5 × 60 cm) equilibrated with buffer B.

The desalted solution from the anion exchange column was applied to a phenyl agarose hydrophobic interaction affinity column (0.8 × 5 cm) equilibrated with 3 column vol of 1.5 M ammonium sulfate in buffer B. Proteins were eluted with a linear gradient of ammonium sulfate from 1.5 M to 0 M at a flow rate of 40 ml h^{-1}. Fractions (2 ml) containing NAD^+-IDH activity were collected, pooled, and applied to a Sephadex G-100 (1.5 × 50 cm) size exclusion column equilibrated with buffer B at a flow rate of 12 ml h^{-1}. Fractions (2.5 ml) with NAD^+-IDH activity were combined and stored with 40% glycerol at -20°C prior to use.

Enzyme assay NAD^+-IDH was assayed by the modified method of Kornberg (1955) with the basic assay mixture containing 1 mM NAD^+, 1 mM *threo*-DL-isocitrate, 5 mM $MgCl_2$, 1 mM AMP, and 150 mM Tris-acetate buffer at pH 7.6, unless otherwise indicated. Activity of the enzyme was determined by monitoring the increase in absorbance at 340 nm resulting from the reduction of NAD^+ to NADH at 25°C for 2 min. Concentrated enzyme solutions were sufficiently diluted with buffer B to obtain a linear response. The assay mixture, without enzyme, was preincubated for 5 min at 25°C and the reaction was then started by the addition of 50 μl of enzyme preparation. One unit of enzyme activity was defined as the amount of enzyme required to produce 1 μmol of NADH min^{-1}. Specific activity was expressed as units mg^{-1} protein. Any significant interference of enzyme activity by other compounds possibly included in the reaction mixture was not detected when the assay was performed without substrates (isocitrate and/or NAD^+) and with heat-deactivated enzyme solution. From the comparison of enzyme activity with standard D- and L-isomers of isocitrate, only the D-isomer of a racemic mixture was determined to be utilized as substrate by this enzyme. Any significant interference of enzyme activity by the L-isomer was not detected.

Protein measurement Protein content was determined colorimetrically at 595 nm using the Bio-Rad protein reagent according to the method of Bradford (1976), with bovine serum albumine (BSA) as the standard protein.

Determination of lipid content Total lipid content of *P. ultimum* mycelium was estimated from the total fatty acid content determined by gas-liquid chromatography using $C_{23:0}$ as an internal standard (Kim, 1997). The values for each experiment are the means of two treatment replications. Standard deviations between replicates were less than 10%.

Results

Partial purification of NAD$^+$-IDH In previous studies, we prepared *P. ultimum* NAD$^+$-IDH to relatively high purity (211-fold) with a specific activity of 76.2 units/mg protein (Kim *et al.*,1996). However, the purified enzyme was unstable and lost significant activity within a few days at 0°C. Therefore, experiments were carried out with a less-pure enzyme preparation that retained 85% of its activity in the presence of 40% glycerol for up to 10 days at –20°C. Therefore, for this study, NAD$^+$-IDH was purified 50-fold and had a specific activity of 24.9 units/mg protein.

NAD$^+$-IDH activity with fungal growth NAD$^+$-IDH activity was determined over nine days of culture growth where the stationary phase was reached at about three days after inoculation (Fig. 1). The specific activity of NAD$^+$-IDH peaked at 0.825 units/mg protein and fell to 0.452 within the first 24 h of culture growth. Activity declined thereafter to 0.239 units/mg protein at 216 h after inoculation.

Effects of nicotinamide nucleotides NAD$^+$-IDH activity was inhibited 15% by NADH at 0.1 mM but no further inhibition was observed at up to 0.25 mM (Table 1). On the other hand, NADPH inhibited activity by 32% at 0.1 mM and progressively to 57% at up to 0.25 mM. Based on Michaelis-Menten and Lineweaver-Burk plots, inhibition of NAD$^+$-IDH by NADPH appeared to be noncompetitive with isocitrate and mixed-noncompetitive with NAD$^+$.

Effects of nucleotides ADP, ATP, and GTP inhibited NAD$^+$-IDH activity by 32, 37, and 36% at 5 mM concentrations, respectively (Table 1). No inhibition by GMP and AMP was detected. Enzyme activity with 2 mM AMP remained unchanged at saturating (1.0 mM) and non-saturating (0.2 mM) concentrations of isocitrate with non-saturating NAD$^+$ (0.3 mM) (data not shown).

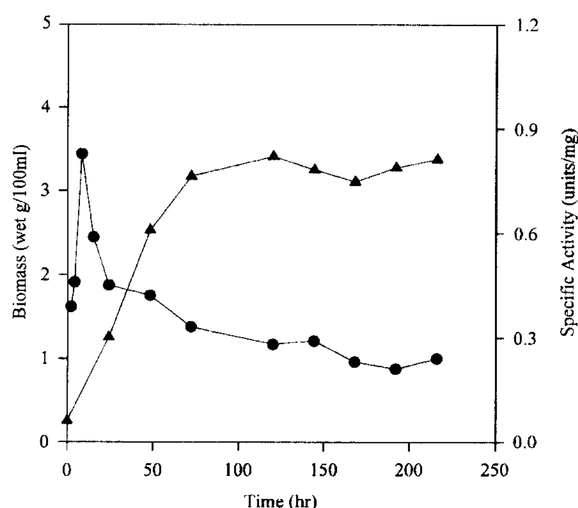


Fig. 1. Growth curve and NAD$^+$-IDH activity of *P. ultimum* as a function of culture development. Biomass (▲) and specific activity (●).

Effects of metabolites In a preliminary experiment, some citric acid cycle and glyoxylate cycle intermediates were tested for their effect on the activity of NAD$^+$-IDH. Five of the metabolites showed 29 to 46% inhibition of activity at the highest concentration tested (10 mM) (Table 1), whereas malate, succinate, sodium glutamate, and pyruvate showed only 4 to 11% inhibition (data not shown). The type of inhibition for the metabolites with the highest inhibitory activity (glyoxylate, citrate, *cis*-aconitate, α-ketoglutarate, and oxalacetate) was determined from Michaelis-Menten and Lineweaver-Burk plots of the activity against isocitrate concentration with varying concentrations of the inhibitor. It was found that oxalacetate and α-ketoglutarate were competitive inhibitors, citrate and *cis*-aconitate were mixed-noncompetitive, and glyoxylate was uncompetitive.

Effects of paired metabolite combinations The five most inhibitory metabolites were combined in pairs at 1 mM each and tested for their effects on NAD$^+$-IDH activity. The combinations of oxalacetate and α-ketoglutarate, glyoxylate, and *cis*-aconitate showed 17% and 24% inhibition, respectively, relative to the control with no metabolites, and other combinations showed only 1% to 10% inhibition (Table 2). However, the glyoxylate/oxalacetate combination inhibited activity by 98%.

Table 1. Effect of metabolic regulators on NAD$^+$-IDH activity.

Regulators	Relative acitivity (%)
Control	100
Nicotine amide nucleotides	
NADH (0.1 mM)	85
NADH (0.25 mM)	90
NADPH (0.1 mM)	68
NADPH (0.25 mM)	43
Nucleotides	
ADP (5 mM)	68
ATP (5 mM)	63
GTP (5 mM)	64
GMP (5 mM)	100
AMP (2 mM)	102
AMP (5 mM)	100
Metabolites (at 10 mM)	
Glyoxylate	57
α-Ketoglutarate	54
Oxalacetate	69
cis-Aconitate	59
Citrate	71

Glyoxylate and oxalacetate alone at 1 mM gave only 4% and 5% inhibition, respectively, and 43% and 31% inhibition at 10 mM.

Effect of glyoxylate and oxalacetate in combination on NAD^+-IDH activity The synergistic inhibition of NAD^+-IDH activity by glyoxylate/oxalacetate was apparent at relatively low concentrations of each inhibitor (Fig. 2). Enzyme activity was reduced by more than 50% with the addition of 80 μM of each inhibitor, and by 90% at 300 μM of each. The addition of AMP did not affect the inhibitory activity by the glyoxylate/oxalacetate combinations (data not shown).

Table 2. Effect of intermediary metabolite combinations on the activity of NAD^+-IDH from *P. ultimum*.

Metabolites (1 mM)[a]	Relative activity (%)
Control	100
Glyoxylate + OAA	2
Glyoxylate + α-ketoglutarate	95
Glyoxylate + Citrate	96
Glyoxylate + *cis*-Aconitate	76
OAA + α-ketoglutarate	83
OAA + Citrate	97
OAA + *cis*-Aconitate	90
α-Ketoglutarate + Citrate	98
α-Ketoglutarate + *cis*-Aconitate	95
Citrate + *cis*-Aconitate	99

[a]Concentration of each metabolite was 1 mM.

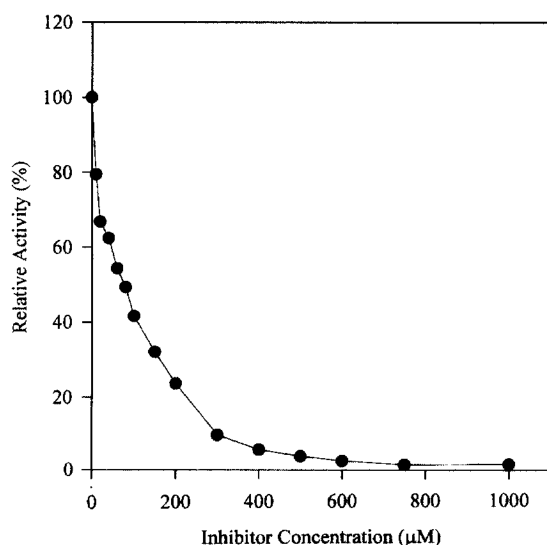


Fig. 2. Effect of oxalacetate/glyoxylate combination in equal amounts as a function of concentration on the activity of NAD^+-IDH.

The effect of glyoxylate and oxalacetate in combination on NAD^+-IDH activity was studied further by varying the amount of one inhibitor and keeping the other constant at 1 mM. In both cases, enzyme activity was most responsive to the lowest concentrations of varied metabolite at 0.01 to 0.1 mM with only slight increases in inhibition up to 0.5 mM (Fig. 3). Inhibition of activity with a fixed amount of oxalacetate and varied glyoxylate was greater than that with a fixed amount of glyoxylate and varied oxalacetate. The ratio of inhibition with varied oxalacetate over glyoxylate increased progressively from just under two to about 12 with increasing concentration of the varied metabolite. At 0.5 mM glyoxylate and 1 mM oxalacetate, only 2% of the original enzyme activity remained. The difference in activity between the two separate combinations was constant at about 30% up to 0.5 mM of varied metabolite concentration. Inhibition of activity with a fixed amount of glyoxylate (1 mM) and varied oxalacetate (0, 10, 50 μM) was competitive against isocitrate determined from Michaelis-Menten and Lineweaver-Burk plots. However, NAD^+-IDH activity with a fixed amount of oxalacetate (1 mM) and varied glyoxylate (0, 5, 20 μM) showed a sigmoidal curve rather than the typical hyperbolic curve (Fig. 4). As the concentration of glyoxylate increased, the sigmoidal shape became more apparent, indicating that glyoxylate plays a major role in this synergistic inhibitory effect on NAD^+-IDH.

Kinetic analysis of the inhibition When used alone, glyoxylate and oxalacetate exhibited uncompetitive and competitive inhibition against isocitrate, respectively. However, when combined together, inhibition was competitive against isocitrate as determined from

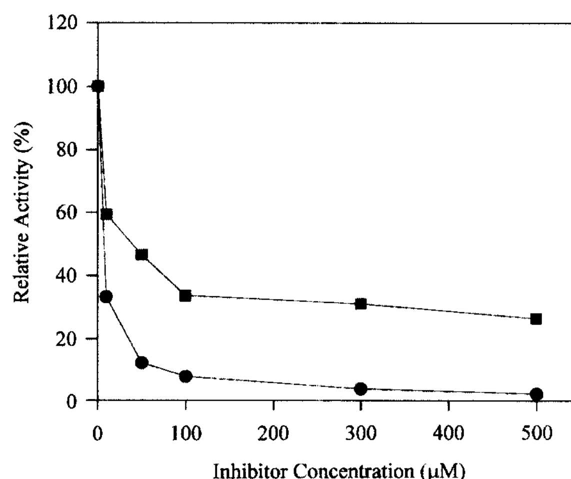


Fig. 3. Effect of glyoxylate and oxalacetate in combination at different concentrations on NAD^+-IDH activity. 1 mM glyoxylate and varied amounts of oxalacetate (■), 1 mM oxalacetate and varied amounts of glyoxylate (●).

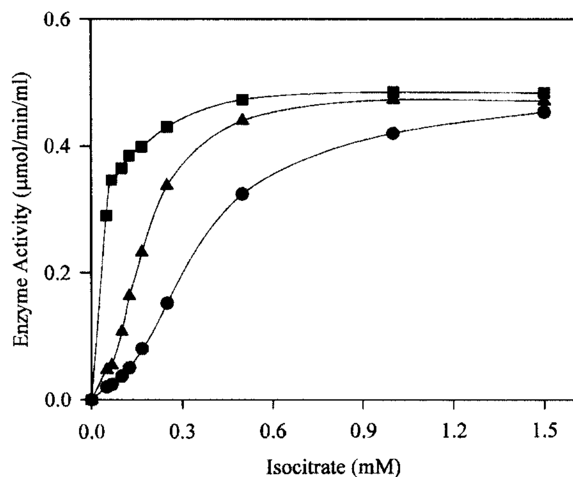


Fig. 4. Effect of oxalacetate/glyoxylate combination at a fixed concentration of oxalacetate (1 mM) and varied concentrations of glyoxylate on NAD^+-IDH activity as a function of isocitrate. Glyoxylate at 0 μM (■), 5 μM (▲) and 20 μM (●).

Michaelis-Menten and Lineweaver-Burk plots. To elucidate the mechanism of the synergistic inhibition by glyoxylate and oxalacetate, the kinetic properties of inhibition were determined from the reciprocal plots of reaction velocity against isocitrate as the substrate (Table 3). Oxalacetate, as a competitive inhibitor against isocitrate, showed a K_i of 2.3 mM. The apparent K_M for isocitrate was 0.045 mM at 1 mM of the inhibitor. The apparent K_M for isocitrate with oxalacetate was 1.5 times higher than that without inhibitor. However, when the uncompetitive inhibitor glyoxylate was combined in a 1:1 ratio with oxalacetate, kinetic values were greatly changed. The K_i with respect to isocitrate was 6 μM, which was 383 times lower than that with oxalacetate alone. The apparent K_M with glyoxylate/oxalacetate at 500 μM each was 116 times and 168 times higher than with and without (control) oxalacetate, respectively.

Effect of glyoxylate/oxalacetate combination on the growth and lipid production of *P. ultimum* The glyoxylate/oxalacetate combination in a 1:1 ratio were added to a 6 d old culture and incubated for an additional 3 days. This treatment had little effect on biomass (Table 4). However, lipid content increased by up to 49% over the control when 3 mM of each metabolite was added, resulting in the increase of total lipid yield (0.94 g/l) by 31% over the control. However, when 2% glucose was added to the culture, total lipid yield was similar (0.93 g/l) to the results above, due to the increase in biomass production (15%), although the increase in total lipid content of the dry mycelia (12%) over the control was less than that induced by the metabolites (49%).

Table 3. Inhibition kinetics of NAD^+-IDH with isocitrate as substrate by oxalacetate and oxalacetate plus glyoxylate.

Kinetics	Control[a]	Inhibitors	
		Oxalacetate	Oxalacetate/ Glyoxylate[c]
K_i (mM)		2.3	0.006
K_M^{APP} (mM)[b]	0.031	0.045	5.2
K_M^{APP}/K_M	1	1.5	167.7

[a] Represents kinetic values without inhibitor.
[b] Values were calculated at 1 mM of inhibitor concentration.
[c] Two inhibitors were combined in equal amounts.

Table 4. Effect of metabolites on the growth and lipid production by *P. ultimum*.

Conditions[a]	Biomass (g/l)[c]	Total lipid	
		(%)[d]	(g/l)
Control[b]	4.7	15.4	0.72
Glyoxylate (1 mM) and OAA (1 mM)	3.5	20.4	0.71
Glyoxylate (3 mM) and OAA (3 mM)	4.1	22.9	0.94
Glucose (2%)	5.4	17.2	0.93
H_2O only	3.4	16.0	0.54

[a] Metabolites were added to the culture after 6 days of incubation followed by additional 3 day incubation.
[b] Fungus was grown for 6 days in the base medium.
[c] Dry weight per liter of culture.
[d] Weight percentage.

Discussion

The regulation of NAD^+-IDH from *P. ultimum* was quite unlike those reported for other fungi including the oleaginous yeast, *R. toruloides* (Botham and Ratledge, 1979; Evans and Ratledge, 1985). Inhibition of the NAD^+-IDH activity by NADPH rather than NADH was unique from other NAD^+-IDH enzymes reported, where NADH gave substantial inhibition of the enzyme (Coultate and Dennis, 1969; Gabriel and Plaut, 1984; Evans and Ratledge, 1985; Mcintosh and Oliver, 1992).

Dependence of IDH activity on AMP has been described as a key factor in the oleaginicity of the yeast *Rhodosporidium toruloides* CBS 14 under nitrogen-limiting conditions, whereby AMP is depleted causing citrate to accumulate and move from the mitochondria into the cytosol, becoming a substrate for fatty acid biosynthesis (Evans and Ratledge, 1984a, 1984b). However, the NAD^+-IDH from *P. ultimum* showed no significant dependence on AMP. Activity of the enzyme

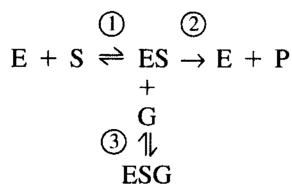
remained unchanged with saturating and non-saturating concentrations of isocitrate. From these results, it was concluded that the *P. ultimum* NAD^+-IDH is not AMP-dependent and AMP is not a key factor in the regulation of this enzyme.

Inhibition of this enzyme by citrate appeared to be different from that of other sources. NAD^+-IDH from the fungus *Blastocladiella emerisonii* was activated by citrate by converting the aggregated enzyme (active) to the monomeric form (inactive) (Le'John *et al.*, 1969). In *Neurospora crassa*, this enzyme was activated by citrate at pH 7.6 but not at pH 6.4 (Sanwal and Stachow, 1965). Citrate activated NAD^+-IDH from baker's yeast without AMP, whereas, when activated by AMP, citrate inhibited activity (Gabriel and Plaut, 1991). Citrate at 5 mM inhibited NAD^+-IDH from *Rhodosporidium toruloides* CBS 14 by 32% (Evans and Ratledge, 1985). NAD^+-IDH from *P. ultimum* was inhibited at 10 mM citrate by 29%.

The combination of glyoxylate and oxalacetate was a powerful inhibitor of *P. ultimum* NAD^+-IDH and this inhibition seemed at first observation to be for NAD^+-specific isocitrate dehydrogenase even though $NADP^+$-specific IDH from other sources including fungus (Takao *et al.*, 1986), a protozoan (Marr and Weber, 1969), a bacterium (Shiio and Ozaki, 1968), and plants (Satoh, 1972) were reported to be inhibited by this metabolite combination.

According to kinetic analysis, inhibition of NAD^+-IDH by oxalacetate was competitive against isocitrate. Under the condition where oxalacetate is present alone as an inhibitor, dissociation of the enzyme-OAA complex might be expected to be favored due to the relatively high K_i value (2.3 mM) with oxalacetate compared to the K_M value with substrate (0.031 mM). Therefore, the substrate becomes more competitive against oxalacetate for binding at the active site. This results in weak inhibition of enzyme activity by oxalacetate alone at even relatively high concentrations (31% at 10 mM).

On the other hand, glyoxylate inhibits NAD^+-IDH activity uncompetitively, which can be described as follows:



where E, S, G, and P are enzyme, isocitrate, glyoxylate, and product, respectively. In this reaction, glyoxylate binds to the enzyme-substrate complex at a site that might be exposed by a conformational change of enzyme structure through the binding of substrate at the active site. Reaction (3) is reversible and might be expected to favor dissociation based on the fact that even at a relatively high concentration of glyoxylate (10 mM), inhibition of enzyme activity was moderate (43%).

When oxalacetate was combined with glyoxylate in the same ratio and examined together in the reaction, the kinetic values given in Table 3 represented dramatically reduced K_i and increased apparent K_M values of oxalacetate when compared to those with oxalacetate alone, indicating that the affinity of this inhibitor for the active site was greatly increased. This explains the increased NAD^+-IDH inhibition by the metabolite combination over oxalacetate alone. A possible explanation for this enhanced inhibition by the combination of two metabolites is that not only the enzyme-substrate complex but also the enzyme-oxalacetate complex causes conformational change in the enzyme such that glyoxylate is able to bind to the enzyme-oxalacetate complex to build up the enzyme-oxalacetate-glyoxylate complex and enzyme-substrate-glyoxylate as well. However, the binding affinity of the enzyme-oxalacetate for glyoxylate is assumed to be greatly higher than that of enzyme-substrate resulting in that at a relatively low concentration of substrate, most of the enzyme exists in the relatively stable form of enzyme-oxalacetate-glyoxylate complex, causing the synergistic inhibition of NAD^+-IDH. The fact that the K_i value of oxalacetate alone was greatly decreased according to the significantly reduced enzyme activity by the addition of glyoxylate in the reaction mixture supports this explanation.

Of the two metabolites, oxalacetate is believed to be the key inhibitor and glyoxylate the enhancer of oxalacetate inhibitory action. This is based on the fact that, at a relatively high fixed concentration of glyoxylate and varied low concentration of oxalacetate, inhibition was competitive typical of inhibition at a 1:1 ratio of the materials and oxalacetate alone. On the other hand, at concentrations of varied low glyoxylate and fixed high oxalacetate, inhibition represented a sigmoidal shape. However, the reason why glyoxylate enhanced the inhibition of enzyme activity by only oxalacetate but not α-ketoglutarate, another competitive inhibitor against isocitrate, was uncertain although one probable explanation for this is that α-ketoglutarate can bind to the enzyme at the active site, but it cannot carry out the successful conformational change of enzyme structure to expose another binding site for glyoxylate. Formation of the ternary complex, namely the enzyme-oxalacetate-glyoxylate complex, was suggested to describe the concerted inhibition of $NADP^+$-IDH by combination of oxalacetate and glyoxylate although glyoxylate and oxalacetate showed competitive and mixed-type noncompetitive inhibition instead of uncompetitive and competitive inhibition, respectively (Shiio and Ozaki, 1968).

The regulation of NAD^+-IDH from *P. ultimum* is complex (Fig. 5) and is different from that of *R. toruloides*

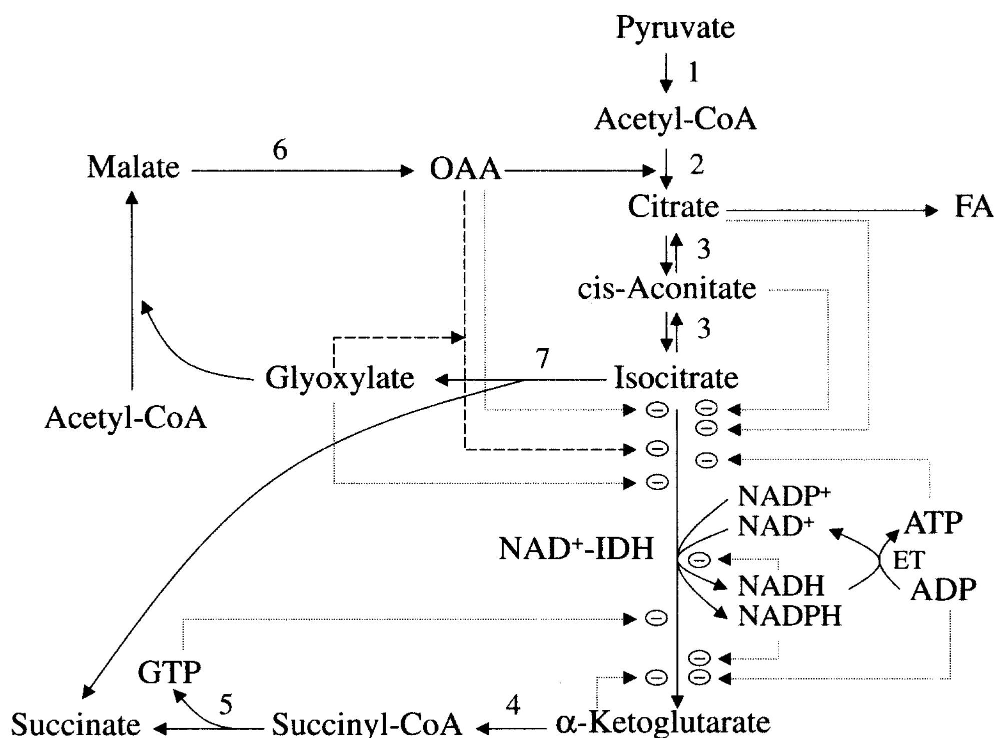


Fig. 5. Regulatory scheme of NAD$^+$-IDH from *P. ultimum*. Dotted lines, inhibition; dashed lines, synergistic inhibition. ET, electron transport system; FA, fatty acid; 1, pyruvate dehydrogenase; 2, citrate synthase; 3, aconitase; 4, α-ketoglutarate dehydrogenase; 5, succinyl-CoA synthetase; 6, malate dehydrogenase; 7, isocitrate lyase.

where the corresponding enzyme is AMP-dependent (Botham and Ratledge, 1979; Evans and Ratledge, 1985). A possible explanation for the regulation of NAD$^+$-IDH in *P. ultimum* based on the results of this study may be as follows: As mycelial growth decreases with culture age, continued uptake and metabolism of glucose through glycolysis and the initial steps of the TCA cycle result in the build up of isocitrate; therefore, NAD$^+$-IDH would be expected to function at maximum activity. Isocitrate would equilibrate with citrate and also become a substrate for isocitrate lyase resulting in the production of glyoxylate. At a relatively constant level of oxalacetate, glyoxylate would become the primary regulatory factor for modulating the activity of the *Pythium* NAD$^+$-IDH. Citrate would be transported from the mitochondria to the cytosol where it would be metabolized to lipid. Although *P. ultimum* has the potential to produce a relatively high amount of lipid, one reason for the limited partitioning of carbon to oil may be the decrease in substrate due to the breakdown of isocitrate.

The fact that NAD$^+$-IDH was inhibited by various metabolites indicates that it may occupy an important position in the intracellular distribution of carbon, and therefore possibly lipid accumulation in *P. ultimum*, suggesting that there may be several possible ways to manipulate this enzyme for enhancing lipid accumulation in this fungus. The result that lipid production of this fungus was promoted by the addition of combined inhibitory metabolites supports this suggestion. However, there are still certain points to be studied, for example, although lipid production was promoted by those inhibitory metabolites, the value of total lipid content was only 22.9%, which is not relatively high compared to other oleaginous microorganisms.

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