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NADP-specific isocitrate dehydrogenase from the citric acid-accumulating fungus *Aspergillus niger*

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NADP-specific isocitrate dehydrogenase [*threo*-D_s-isocitrate: $NADP^+$ oxidoreductase (decarboxylating), EC 1.1.1.42] was purified 200–300-fold from the citric acid-accumulating fungus *Aspergillus niger*. The enzyme consists of a single polypeptide chain with a molecular mass of 60 ± 4 kDa and has a pI of 5.9 ± 0.2. Only a single enzyme protein was found, although the enzyme appears to occur both in the mitochondrion and in the cytoplasm. Growth on organic acids as carbon sources or on NO_3^- as nitrogen source led to increased activities, whereas the presence of amino acids led to lower activities. The enzyme exhibits hyperbolic kinetics with respect to its substrates isocitrate and $NADP^+$. Mn^{2+} and Mg^{2+} are obligatory for enzyme activity. The enzyme is inhibited by its products α-oxoglutarate and NADPH. Among various metabolites, ATP and citrate appear to inhibit the enzyme at a concentration considered to occur intracellularly. In both cases, however, the mechanism is a removal of the metal ion cofactor from the substrates. It is concluded that under physiological conditions, where the Mg^{2+} content is around 10 mM, the observed inhibition by ATP or citrate is of poor regulatory significance.

INTRODUCTION

NADP-specific isocitrate dehydrogenase [NADP-IDH; *threo*-D_s-iosocitrate:$NADP^+$ oxidoreductase (decarboxylating), EC 1.1.1.42] is known to occur in higher eukaryotic tissues in two distinguishable forms, namely the heart type, which mainly occurs in the mitochondria, and the liver type, which mainly occurs in the cytosol (Fatania & Dalziel, 1980; Carlier & Pantaloni, 1973). These enzymes have been shown to be under independent genetic control (Lowenstein & Smith, 1962) and to differ in their physical and enzymic properties (Uhr *et al.*, 1974; Ehrlich & Colman, 1976; Carlier & Pantaloni, 1976*a*,*b*). In contrast, the enzyme from simple eukaryotes has so far been little investigated (Ingebretsen, 1975; Dedhia *et al.*, 1979).

The filamentous fungus *Aspergillus niger* is known for its ability to accumulate large quantities of citric acid when grown under certain defined conditions (Röhr *et al.*, 1983). This organism has been reported to contain only very low amounts of NAD-specific isocitrate dehydrogenase [*threo*-D_s-isocitrate:NAD^+ oxidoreductase (decarboxylating), EC 1.1.1.41] and an NADP-IDH, which is distributed both in the cytosolic and in the mitochondrial compartment (Mattey, 1977). Inhibition of the mitochondrial isocitrate dehydrogenase by citrate has been claimed (Mattey, 1977) to be the triggering event for citric acid accumulation. Meixner-Monori *et al.* (1985) have, however, suggested that oxaloacetate inhibition of the α-oxoglutarate dehydrogenase multienzyme complex [α-oxoglutarate:lipoate oxidoreductase (acceptor-acetylating), EC 1.2.4.2] is the initial event leading to citric acid accumulation.

NADP-specific isocitrate dehydrogenase from *A. niger* has hitherto been neither purified nor studied with respect to its kinetic and regulatory properties. The present paper describes this.

MATERIALS AND METHODS

Materials

Substrates, coenzymes and auxiliary enzymes were obtained from either Boehringer, Mannheim, Germany, or Sigma Chemical Co., St. Louis, MO, U.S.A. Triazine dyes were given by ICI Chemicals, Leicester, U.K. Reactive Blue–agarose was from Sigma. All other chemicals were of analytical grade.

Culture conditions and extraction of the enzyme

Aspergillus niger B60 (Röhr *et al.*, 1979) was used throughout these studies. The organism was kept on potato dextrose/agar slants and, unless otherwise stated, cultivated in 1% (w/v) glucose medium as described previously (Ma *et al.*, 1981). Cell free extracts were prepared from freshly harvested mycelia by the procedure described by Meixner-Monori *et al.* (1985), which involves grinding under liquid N_2 and subsequent ultrasonication. The extractant was 50 mM-Tris/HCl, pH 7.8, containing 1 mM-$MnCl_2$, 5 mM-citrate and 5 mM-dithiothreitol. The homogenate was centrifuged at 14000 *g* (25 min, 4 °C), and the supernatant was used on the same day for purification of the enzyme. Up to 50 ml of extract could be prepared at one time.

Analytical methods

Protein was determined by the Coomassie Blue method (Bradford, 1976). Enzyme activity was measured by

Abbreviation used: NADP-IDH, NADP-specific isocitrate dehydrogenase.

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monitoring the appearance of NADPH at 340 nm in a Pye–Unicam UV/Vis spectrophotometer equipped with an external recorder at a chart speed of 1 cm/min. Temperature was kept at 28 °C. The reaction system contained, in a total volume of 2 ml: 50 mM-potassium phosphate, pH 7.0; 0.2 mM-$NADP^+$; 0.18 mM-DL-isocitrate; 0.5 mM-$MnCl_2,4H_2O$ or 3.3 mM-$MgCl_2,6H_2O$; and enzyme, sufficient to produce an increase in A_{340} between 0.02 and 0.15 per min. The reaction was always started by the addition of isocitrate. The recorded curves were extrapolated to the time of addition of the enzyme, and the tangents to the curves at this time were taken as the initial velocities. Unless otherwise specified, 1 unit of NADP-IDH activity corresponds to the formation of 1 μmol of NADPH/min under the assay conditions. Specific activities are given as units/mg of protein.

Other enzymes were assayed by the methods given in parentheses: citrate synthase, EC 4.1.3.7 (Kubicek & Röhr, 1980); α-oxoglutarate dehydrogenase (Meixner-Monori *et al.*, 1985); glucose-6-phosphate dehydrogenase, EC 1.1.1.49 (Niehaus & Dilts, 1984); NAD:malate dehydrogenase, EC 1.1.1.37 (Ma *et al.*, 1981).

Subcellular localization of *A. niger* enzymes

This was done by the method of Osmani & Scrutton (1983), which involves enzymic weakening of the fungal cell wall and subsequent breaking of the cells by nitrogen cavitation, with the following modifications: (*a*) enzymic pretreatment was carried out by suspending 1 g of mycelium (wet wt.) in 10 ml of 0.2 M-sodium phosphate buffer, pH 6.4, containing 0.6 M-mannitol and 4 mg of Novozyme 234/ml (Novo Industries, Bagsvaerd, Denmark), and gently agitating for 30 min at room temperature; (*b*) the buffer used for N_2 cavitation was 10 mM-Tris/HCl, pH 7.5, containing 2 mM-$MgCl_2$, 1 mM-EDTA, 0.25 M-mannitol, 0.1 mM-dithiothreitol and 4 μM-phenylmethanesulphonyl fluoride; differential centrifugation was performed essentially as described by Osmani & Scrutton (1983). The individual fractions were kept at 4 °C and analysed within 3 h.

Electrophoresis

Polyacrylamide-gel electrophoresis under denaturing conditions (SDS) was carried out in a conventional vertical slab-gel apparatus in the system of Laemmli (1970). Proteins were silver-stained by the procedure of Morrisey (1981).

Gel filtration

Determination of native molecular mass was carried out by gel filtration on a column (16 mm × 380 mm) of Sephadex G-150, previously equilibrated in buffer A [5 mM-potassium citrate, pH 7.8, containing 1 mM-$MnCl_2,4H_2O$ and 10% (w/v) glycerol]. The position of NADP-IDH was verified by measurement of enzyme activity; recovery was 85–90% of applied activity. The column was calibrated with commercial marker proteins: yeast alcohol dehydrogenase (158 kDa); bovine serum albumin (66.2 kDa); ovalbumin (45 kDa); carbonic anhydrase (29 kDa).

Enzyme purification

Crude cell-free extract (150 ml) was fractionated by $(NH_4)_2SO_4$ precipitation between 46 and 70% saturation as described by Ramakrishnan & Martin (1955). The precipitate was dissolved in 0.1 vol. of buffer A and desalted on a Pharmacia PD-10 ready-to-use column. The enzyme-containing eluate was applied to a column of Reactive Blue–agarose (15 mm × 125 mm) at a flow rate of 16 ml/h at 4 °C. By this method, originally introduced by Balamir (1983), the enzyme is eluted in a somewhat retarded position and is thus separated from most of the other proteins which are eluted in the void volume. The fractions containing more than 30% of activity were pooled, passed through 0.22 μm-pore-size membrane filters, and subjected to fast protein liquid ion-exchange chromatography with Pharmacia FPLC equipment. Mono Q anion exchanger was equilibrated with buffer A at a flow rate of 1 ml/min. When no more protein was eluted from the column, a linear gradient of 0–0.5 M-KCl in buffer A was applied, which led to elution of

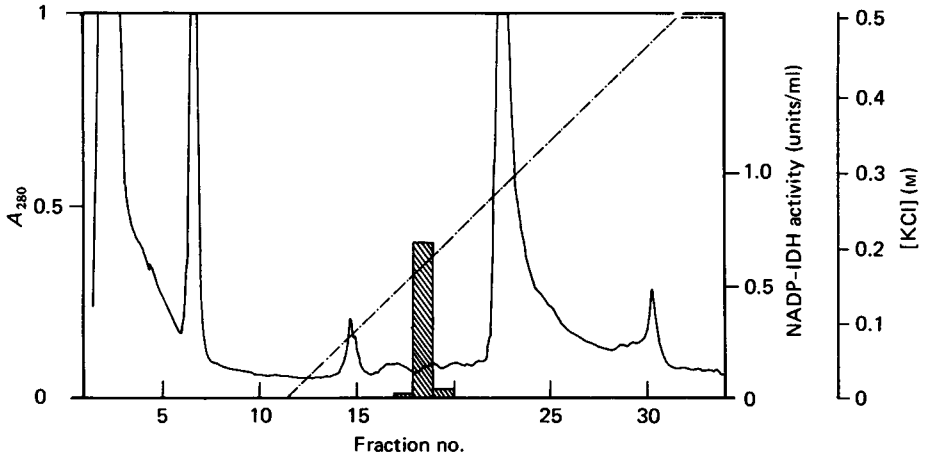

Fig. 1. Fast protein liquid anion-exchange of NADP-IDH on Mono Q chromatography

Conditions were as described in the Materials and methods section. Fractions (2 ml) were collected and assayed for activity (■). Protein (——; A_{280}) was monitored automatically; –·–·–, KCl gradient.

NADP-IDH in a single fraction at a position clearly separated from most of the other proteins (Fig. 1). The enzyme was again desalted on a PD-10 column and used for the kinetic investigations, or stored at −20 °C until analysis.

RESULTS

Formation and subcellular localization of NADP-IDH in *Aspergillus niger*

Table 1 documents the effect of different conditions of growth on the mycelial activity of *A. niger* NADP-IDH: typically, increased activities were observed either on growth on carbon sources derived from the citric acid cycle (e.g. citrate) or when NO_3^- was used as a nitrogen source. On the other hand, the use of amino acids as nitrogen sources led to somewhat lowered activities. These results suggest a mechanism for coarse regulation of NADP-IDH from *A. niger* similar to that proposed for the closely related species *A. nidulans* (Kelly & Hynes, 1982).

Fig. 2 shows the results from subcellular fractionation of *A. niger*: NADP-IDH appeared in both the 14000 *g* debris and the 100000 *g* supernatant. By assaying several other enzymes of known location in *A. nidulans* (Osmani & Scrutton, 1983), evidence was obtained that the 14000 *g* pellet contained mitochondrial enzymes, whereas the 100000 *g* supernatant contained cytoplasmic enzymes. Contamination by mitochondrial enzymes (as shown by distribution of citrate synthase), owing to partial breakage of mitochondria, probably did not exceed 10%; from these data it is concluded that NADP-IDH in *A. niger* is approximately equally distributed between mitochondria and cytoplasm. Coinciding results have previously been reported for *A. nidulans* and acetate-grown *Rhizopus nigricans* (Osmani & Scrutton, 1983, 1985). During growth on media containing either citrate as a carbon source, or NO_3^- as a nitrogen source, elevated activities of NADP-IDH were observed in the cytosolic fraction, whereas the activity in the mitochondrial fraction was unaltered.

Purification of NADP-IDH from *A. niger*

By the method described in the Materials and methods section NADP-IDH from glucose/NH_4^+-grown mycelia of *A. niger* was purified approx. 200–300-fold. Instability of the enzyme, which was initially observed to occur especially at later stages of purification, could be counteracted by the use of Mn^{2+}/citrate-containing buffers, and the inclusion of glycerol after the precipitation step. The method so developed consisted of two main steps, which could be carried out with excellent yield and purification (Table 2). The affinity chromatography on

Table 1. NADP-IDH activity under various growth conditions

Growth was carried out in the medium decribed by Ma *et al.* (1981), in which carbon and nitrogen sources were replaced by the additions given in the Table. The numbers in parentheses indicate their respective concentrations in g/litre. The approximate growth rate at the point of harvest was determined by monitoring growth by dry-weight measurements. All cultures were grown until approximately half of the carbon source was consumed.

Treatment medium	Specific activity (μmol/min per mg of protein)	Growth rate (h^{-1})
Glucose/NH_4^+ (10/5)	0.125	0.150
Glucose/NO_3^- (10/8)	0.175	0.028
Citrate/NH_4^+ (10/5)	0.192	0.020
Citrate/NO_3^- (10/8)	0.210	0.018
Glucose/peptone (10/5)	0.025	0.200
Glucose/glutamate (10/13.2)	0.076	0.100
Glucose/NH_4^+ (150/5)	0.088	0.080

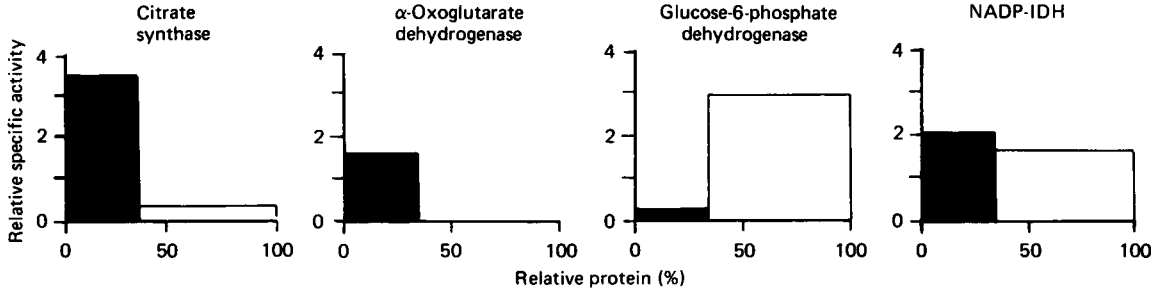

Fig. 2. Subcellular distribution of various enzymes in a cell-free extract of *A. niger*

The data are plotted as described by Osmani & Scrutton (1983), except that 14000 *g* supernatants and 100000 *g* supernatants are combined (several experiments had shown that the activity in the 14000 *g* supernatant was always negligible with the enzymes assayed). ■, 14000 *g* debris; □, combined supernatants.

Table 2. Summary of purification of NADP-IDH from *A. niger* mycelium

Mycelia were harvested from glucose/NH_4^+ medium after approx. 80–90% of the carbon source had been consumed. Although this resulted in a lower specific activity, the total amount of mycelium and thus NADP-IDH activity was greater. Activities cited were assayed as described in the Materials and methods section, within 30 min after completion of a purification step. The summary of purification given is representative of at least five separate trials.

	Total volume (ml)	Total activity (units)	Total protein (mg)	Specific activity (units/mg)	Yield (%)	Purification (-fold)
Cell-free extract	150	22.5	440	0.051	100	1
$(NH_4)_2SO_4$ fractionation followed by Sephadex G-25 desalting	17	22.5	85.7	0.263	100	5.1
Reactive Blue–Agarose	8	20.3	35.1	0.578	90.3	11.3
Fast protein liquid chromatography (Mono Q)	2	19.6	1.3	14.40	87.1	282.3

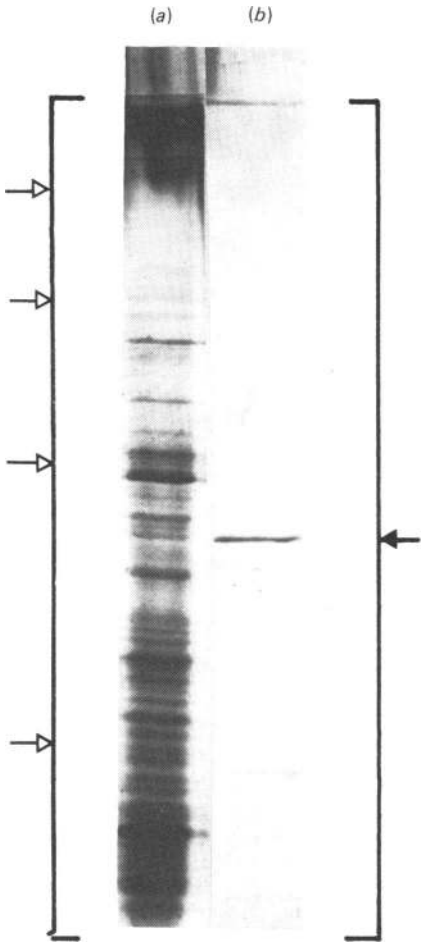

Fig. 3. SDS/polyacrylamide-gel electrophoreses of (*a*) crude *A. niger* extract (10 μg) and (*b*) purified NADP-IDH from *A. niger* (2 μg)

The black arrow indicates the position of NADP-IDH. The other arrows indicate marker proteins (from bottom to top): ovalbumin (45 kDa); bovine serum albumin (66.2 kDa); phosphorylase *b* (92.5 kDa); β-galactosidase (116.5 kDa).

Reactive Blue–agarose could also be replaced by chromatography on 2,5-ADP–Sepharose or Yellow HE 3G–Sepharose, with similar yields and purification. The state of purification so achieved is shown by the SDS/polyacrylamide-gel electrophoresis in Fig. 3, showing one strong band on silver staining, but also a limited number of additional faint bands after prolonged development. The preparation was thus still contaminated with other proteins. The identity of the major band with NADP-IDH was verified by electrophoresis under non-denaturing conditions, where NADP-IDH was found to be associated with the major protein peak. Also, a comparison of the molecular mass of partially purified NADP-IDH (after Reactive Blue–agarose chromatography), 62 ± 7 kDa, with the molecular mass of the major band in SDS/polyacrylamide-gel electrophoresis (60 ± 4 kDa; see Fig. 3) supports this.

It should be especially noted that at no stage of purification was more than a single peak of NADP-IDH detected. The high yield of recovery precludes the possibility that one form of NADP-IDH might have become lost during purification, since subcellular fractionation has shown that the enzymes from both compartments had approximately equally high activities. Also proteolysis during early stages in extract preparation and purification is unlikely, since freshly prepared cell-free extracts also produced only one peak of activity on fast protein liquid ion-exchange chromatography or isoelectric focusing. Furthermore, kinetic investigations on the enzyme (see below) yielded essentially similar results with crude extracts from glucose- or citrate-grown mycelia or with the purified enzyme. It thus appears that, despite the distribution of NADP-IDH in *A. niger* mitochondria and cytoplasm, this fungus contains only a single NADP-IDH protein.

The pI of partially purified NADP-IDH was found to be 5.9 ± 0.2 on isoelectric focusing.

Kinetic and regulatory properties of NADP-IDH from *A. niger*

Initial-velocity substrate kinetics were examined with $NADP^+$ or isocitrate as the variable substrate in the presence of either Mn^{2+} or Mg^{2+} as cofactor. In all cases, families of straight lines were observed which intersected at the abscisa. The K_m values obtained from linear replots of the intercepts of $1/v$ versus 1/[isocitrate] and $1/v$ versus 1/[$NADP^+$] plotted against 1/[$NADP^+$] or 1/[isocitrate] respectively were: (at 0.5 mM-Mn^{2+}) isocitrate 0.077 ± 0.011 mM, $NADP^+$ 0.020 ± 0.007 mM; (at 3.3 mM-Mg^{2+}) isocitrate 0.079 ± 0.013 mM; $NADP^+$ 0.013 ± 0.003 mM.

When the concentration of Mg^{2+} or Mn^{2+} was varied

Table 3. Influence of metabolic intermediates on NADP-IDH from *A. niger*

Experiments were carried out in the presence of 0.053 mM-isocitrate, and all values (means ± S.D. from at least three separate experiments) are given as percentages of the activity in the absence of the added metabolite being investigated.

Intermediate	Concn. (mM)	Metal cofactor Mn^{2+} (0.5 mM)	Metal cofactor Mg^{2+} (3.3 mM)
–(Control)	–	100±8	100±8
Citrate	1	91±8	80±6
	5	46±5	45±5
cis-Aconitate	1	100±9	100±7
Glutamate	5	98±8	98±8
	1	104±9	100±9
Oxaloacetate	1	90±7	85±7
	5	43±4	31±5
Glyoxalate	1	101±7	100±9
	5	100±8	98±8
Oxaloacetate + glyoxalate	0.2 each	9±3	6±3
	0.05 each	30±4	39±4
Oxalate	1	102±8	87±9
	5	93±8	63±7
CoA	1.6	87±7	85±6
ATP	1	58±5	35±6
	5	12±4	30±5
ADP	5	67±6	55±6
AMP	5	91±8	93±8

at fixed concentrations of isocitrate and $NADP^+$, sigmoidal responses were obtained. A calculation of free and chelated isocitrate and $NADP^+$ concentrations under these conditions (by using the complex stability constants given by Kuchel *et al.*, 1980) showed that the concentrations of free isocitrate increased at low metal ion concentration. Separate experiments showed that NADP-IDH did not undergo inactivation by dilution in the assay system, which is prevented by isocitrate or bivalent metal ion–isocitrate; also an elevation of the metal ion concentration at a fixed isocitrate concentration did not inactivate the enzyme. The disproportionate decrease in activity concomitant with the increase in free isocitrate is thus consistent with the assumption of inhibition by free isocitrate of NADP-IDH from *A. niger*. Inhibition of pig heart NADP-IDH by free isocitrate has been observed (Colman, 1972).

The pH optimum of NADP-IDH was rather broad, 85% of maximal activity occurring between pH 6 and 8 when assayed with Mn^{2+} as metal cofactor. With Mg^{2+}, activity declined more rapidly at the acid side of the optimum curve (40% of maximal activity at pH 6.5). This decrease in activity was due to a decrease in $V_{max.}$, whereas the K_m for Mn–isocitrate or Mg–isocitrate remained unchanged within the standard error of the determination (77 ± 16 μM; pH 6.3–7.8).

Among several metabolites related to the activity of the tricarboxylic acid cycle and known to occur in mitochondria, the following were found to be inhibitory (Table 3): adenine nucleotides, NADPH, citrate, α-oxoglutarate, and oxaloacetate plus glyoxalate.

Inhibition by NADPH was found to be competitive with $NADP^+$, yielding a corresponding K_i of 30 ± 8 μM. Since our interest is in regulation, we have also examined the effect of NADPH as an integral part of the total nicotinamide dinucleotide phosphate concentration ('anabolic reduction charge'; Andersen & Van Meyenburg, 1976). The results for several total $NADP^+$ + NADPH concentrations are given in Fig. 4. These results indicate

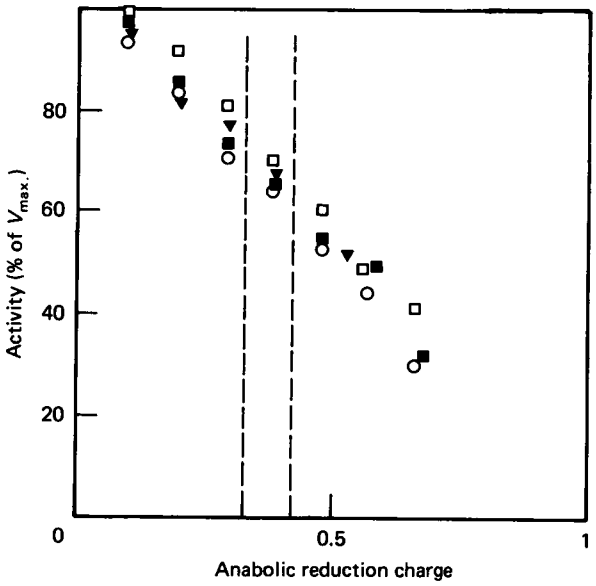

Fig. 4. Inhibition of NADP-IDH by increasing values of the anabolic reduction charge ([NADPH]/[$NADP^+$] + [NADPH]) at several total concentrations of nucleotides

Total concn. of $NADP^+$ + NADPH: □, 0.2 mM; ○, 0.15 mM; ▼, 0.09 mM; ■, 0.045 mM. Activities are given as percentage of activity in the absence of NADPH. The dashed lines bound the values determined for the 'anabolic reduction charge' in *A. niger* (Führer *et al.*, 1980).

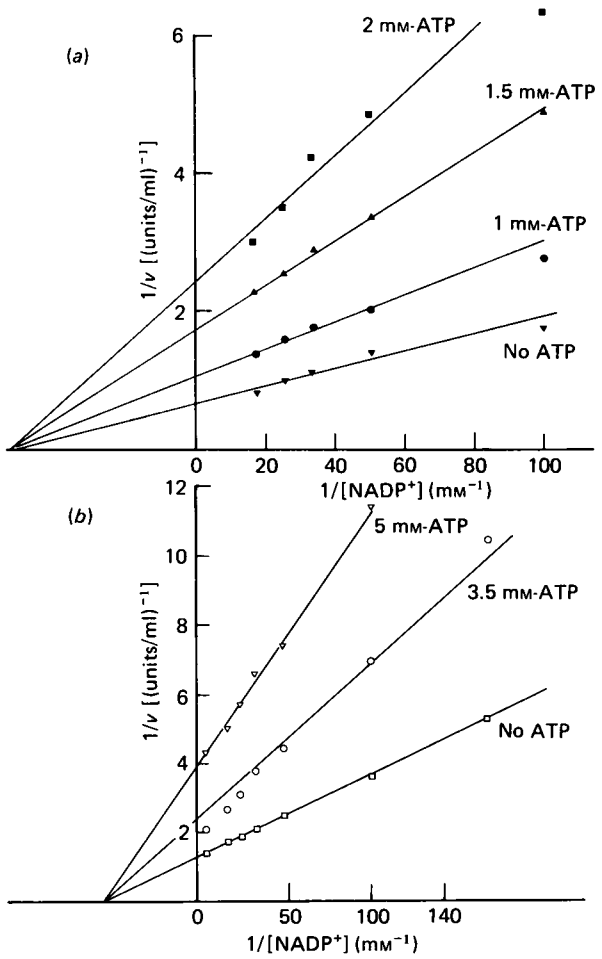

Fig. 5. Lineweaver–Burk plot of inhibition of NADP-IDH by ATP in the presence of (*a*) 0.5 mM-Mn^{2+} or (*b*) 3.3 mM-Mg^{2+}

Other conditions used were: 0.07 mM-isocitrate; 10 mM-Hepes buffer, pH 7.3.

that, under physiological conditions of the anabolic reduction charge (Führer *et al.*, 1980), NADP-IDH should be inhibited by approx. 35%. Care must, however, be applied to this interpretation, since Führer *et al.* (1980) have only measured the total cellular $NADP^+ + NADPH$ concentration, and mitochondrial values of the anabolic reduction charge are still unknown in *A. niger*.

α-Oxoglutarate was found to be a competitive inhibitor of NADP-IDH with respect to isocitrate (K_i 1.3 mM, with Mn^{2+} as cofactor).

ATP and GTP, and also ADP and AMP (but to lesser extents), were also able to inhibit NADP-IDH from *A. niger*. This cast doubt on the biological significance of the data. Lineweaver–Burk plots indicated a non-competitive inhibition (Figs. 5*a* and 5*b*), but when the data were plotted in a Dixon plot non-linearity became evident. This means that, at higher ATP concentrations, activity is lower than expected, which could be interpreted if ATP, by acting as a chelator for Mg^{2+} or Mn^{2+}, removes these metal ions from the substrates isocitrate and $NADP^+$. By using published complex stability constants (Cantore & Passeron, 1982; Kuchel *et al.*, 1980) it could actually be calculated that, at high ATP concentrations, the proportion of Mn^{2+}– or Mg^{2+}–isocitrate and Mn^{2+}– or Mg^{2+}–NADP is very low. Further support for an inhibitory action of ATP on NADP-IDH by acting as a chelator of the necessary metal ions is shown in Figs. 6(*a*) and 6(*b*): inhibition of NADP-IDH by ATP at fixed concentrations of isocitrate and NADP can be modulated by changing the concentration of the metal ion. Furthermore, NADP-IDH was inhibited by pyrophosphate and nucleotides containing bases other than adenine (CTP, GTP). We thus ascribe the inhibitory action of ATP to its ability to form stable complexes with Mn^{2+} and Mg^{2+}, and so to remove these metal ions from NADP-IDH and its substrates.

Citrate also inhibited NADP-IDH of *A. niger* strongly, although the K_i values obtained during this study were approx. 10-fold higher than reported by Mattey (1977). Inhibition by citrate was competitive with isocitrate (Figs.

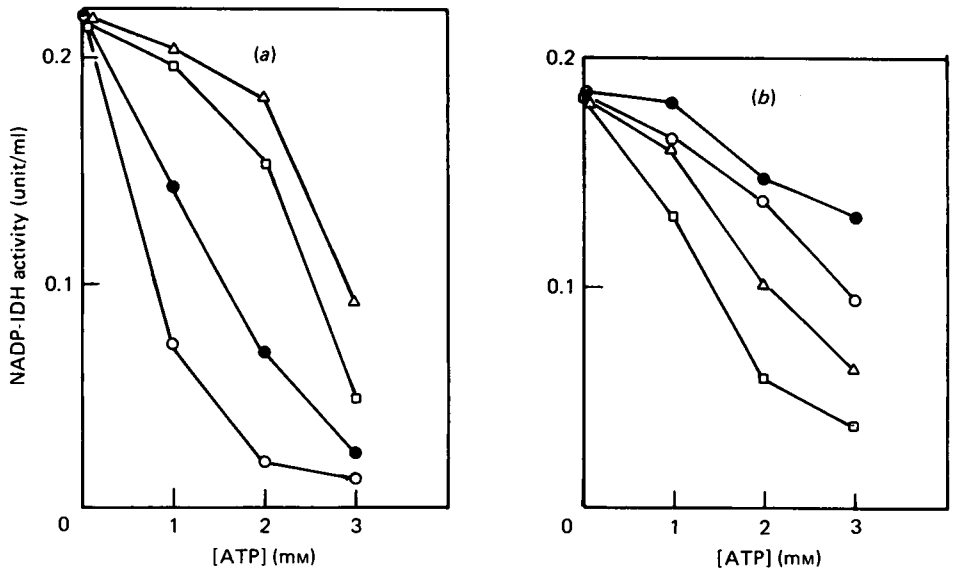

Fig. 6. Inhibition of NADP-IDH from *A. niger* by ATP at various fixed concentrations of metal ion

(*a*) Mg^{2+}: ○, 5 mM; ●, 10 mM; □, 20 mM; △, 30 mM. (*b*) Mn^{2+}: □, 0.5 mM; △, 2 mM; ○, 5 mM; ●, 10 mM. All other conditions were as described in the legend to Fig. 5.

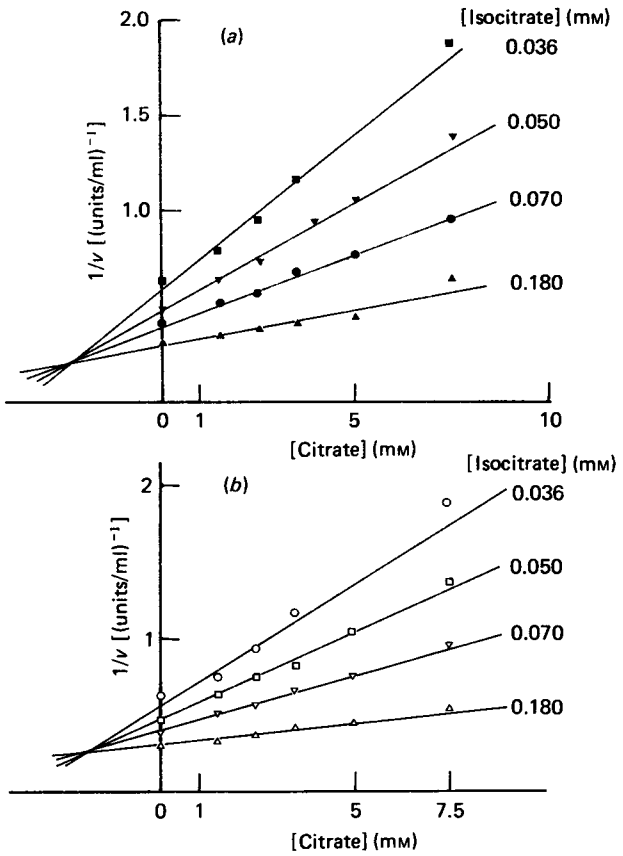

Fig. 7. Dixon plot of citrate inhibition of NADP-IDH in the presence of (*a*) 0.5 mM-Mn^{2+} and (*b*) 3.3 mM-Mg^{2+} as cofactor

Other conditions were: 10 mM-Hepes buffer, pH 7.3; 0.1 mM-$NADP^+$. Respective isocitrate concentrations are indicated in the Figure.

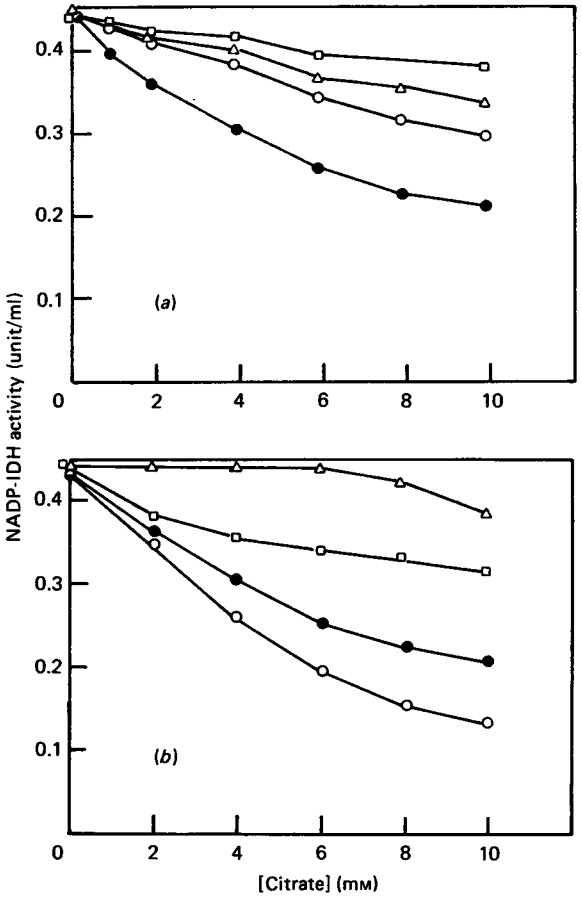

Fig. 8. Inhibition of NADP-IDH from *A. niger* by citrate at various concentrations of metal ion

(*a*) Mg^{2+}: ●, 5 mM; ○, 10 mM; △, 20 mM; □, 30 mM. (*b*) Mn^{2+}: ○, 0.5 mM; ●, 2 mM; □, 5 mM; △, 10 mM. All other conditions were as described in the legend to Fig. 7.

7*a* and 7*b*). An interpretation of this result is, however, complicated by the ability of citrate to chelate Mn^{2+} or Mg^{2+} and so to decrease the concentration of the substrate for NADP-IDH. Unfortunately, experiments in which the concentration of metal ion–isocitrate would be kept constant at different citrate concentrations are impossible, owing to the inhibitory action of free isocitrate (see above). However, support for an action of citrate largely by chelating the metal ion comes from experiments in which isocitrate and $NADP^+$ were kept constant, but citrate was varied at several fixed concentrations of metal ions (Figs. 8*a* and 8*b*). It therefore seems that the inhibitory action of citrate on NADP-IDH from *A. niger* is due to its complex-forming ability with Mn^{2+} and Mg^{2+}.

DISCUSSION

Although the properties of NADP-IDH from higher eukaryotic tissues and also various bacteria are relatively well documented (Howard & Becker, 1970; Charles, 1970; Barrera & Jurtshuk, 1970; Reeves *et al.*, 1972; Self *et al.*, 1973; Carlier & Pantaloni, 1973, 1976*a*,*b*, 1978; Reynolds *et al.*, 1978; Dalziel *et al.*, 1978; Farrell, 1980), the enzyme from fungi, particularly filamentous, has not been studied in detail. To the best of our knowledge, NADP-IDH has only been partially purified from *Phycomyces blakesleanus* (Dedhia *et al.*, 1979) and *Blastocladiella emersonii* (Ingebretsen, 1975). NADP-IDH from *A. niger* is thus the first that has been purified almost to homogeneity. The enzyme consists of a single polypeptide chain (60 kDa), was quite unstable and did not exhibit activity in the absence of added metal ions (Mn^{2+}, Mg^{2+}): all these properties show that the enzyme belongs to the 'heart-type' NADP-IDH. With the other fungi so far studied, higher native molecular masses of NADP-IDH have been obtained (88 and 100 kDa), which would rather fit into the classification as a 'liver type'. Whether this is due to experimental differences, or is of taxonomic significance, can, however, only be assessed when the enzyme is further purified from these other fungi.

Localization of NADP-IDH from *A. niger* is another point of interest: coincident with results from other fungi (Osmani & Scrutton, 1983, 1985) and previous studies on *A. niger* (Mattey, 1977), NADP-IDH was found in both cytosolic and mitochondrial fractions. During purification, however, we were unable to detect more than a single peak of NADP-IDH activity. Also, Ingebretsen (1975) and Osmani & Scrutton (1985), working with *B. emersonii* and *Rhizopus nigricans* respectively, detected only a single species of NADP-IDH in cell-free extracts of

these fungi. In the latter case, however, the single enzyme from glucose-grown cells was found to be localized in the mitochondria exclusively. The dual location of the *A. niger* single NADP-IDH is thus an outstanding property which would require further detailed studies, which were, however, beyond the scope of the present investigation.

As indicated above, our interest in NADP-IDH from *A. niger* is due to its potential role in accumulation of citric acid by this fungus, which is made likely by its mitochondrial location (Mattey, 1977).

NADP-IDH has long been claimed to be the decisive step for citric acid accumulation. Originally, Ramakrishnan *et al.* (1955) attributed citrate accumulation to a loss of aconitase and isocitrate dehydrogenase activities, but later La Nauze (1977) clearly disproved this. Mattey (1977) offered the explanation that intracellular citrate itself inhibits mitochondrial NADP-specific isocitrate dehydrogenase and thereby triggers citrate accumulation in *A. niger*. Such a 'feed-forward inhibition' of NADP-IDH has not been reported with the enzyme from other organisms, and could provide a reasonable explanation why only *A. niger* accumulates large amounts of citric acid, although the physiological advantage of this inhibition is obscure. The results from the present paper, however, give little support to a regulatory role of the citrate inhibition, since in kinetic experiments it turned out to be solely the result of metal ion chelation. Since the cellular Mg^{2+} concentration is far higher than that used for the studies on citrate inhibition, citrate might be even less inhibitory *in vivo* (10 mM intracellular Mg^{2+} in *N. crassa*; Barratt & Cook, 1978). Even the K_i values obtained *in vitro* (3.3 mM-Mg^{2+}) are, however, too high (around 2 mM) to account for a triggering of citric acid accumulation, since intracellular citrate at the beginning of citric acid accumulation is around 1 mM (Habison *et al.*, 1983). Bowes & Mattey (1979) have extended the citrate-inhibition model in terms of inhibition occurring only with Mg^{2+} as cofactor, but not with Mn^{2+}, thereby also offering a plausible explanation for suppression of citric acid accumulation by Mn^{2+} ions (Röhr *et al.*, 1983) coincident with their model. This would apparently contradict our results. However, a more detailed inspection of their experimental data showed that they were working at identical concentrations of Mg^{2+} and Mn^{2+} (3.3 mM), the latter being 6 times the concentration used in our study. Since the citrate–Mn complex is more stable than the Mg–citrate complex, and since we have shown that this inhibition is due to metal ion chelation by free citrate, the higher Mn^{2+} concentration used by Bowes & Mattey (1977) should lead to higher activities (and thus less 'inhibition by citrate'). Since *in vivo*, however, the intracellular concentration of Mn^{2+} is 50–100 times lower than that of Mg^{2+} (cf. Barratt & Cook, 1978), the intracellular changes in Mn^{2+} probably have no consequence on the activity of NADP-IDH. A decrease in the rate of isocitrate oxidation *in vivo* by citrate probably only occurs at late stages of citric acid accumulation, when the cellular concentration is around 9 mM (average cellular distribution, without considering compartmentation), which can chelate a large part of the intracellular Mg^{2+} pool (cf. Kubicek & Röhr, 1986).

The results from the present kinetic and regulatory investigations on NADP-IDH from *A. niger* cast doubt on the role of citrate inhibition for citric acid accumulation by this fungus. This, however, is coincident with our model that citric acid accumulates because of an interruption in the tricarboxylic acid cycle by increased concentrations of oxaloacetate, which inhibit α-oxoglutarate dehydrogenase (cf. Röhr *et al.*, 1983; Kubicek & Röhr, 1986; Meixner-Monori *et al.*, 1985).

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REFERENCES

Andersen, K. B. & Van Meyenburg, K. (1976) J. Biol. Chem. **252**, 4151–4156
Balamir, A. (1983) Biochem. Med. **29**, 194–206
Barratt, D. W. & Cook, R. A. (1978) Biochemistry **17**, 1561–1566
Barrera, C. R. & Jurtshuk, P. (1970) Biochim. Biophys. Acta **220**, 416–429
Bowes, I. & Mattey, M. (1979) FEMS Microbiol. Lett. **6**, 219–222
Bradford, M. (1976) Anal. Biochem. **72**, 248–254
Cantore, M. L. & Passeron, S. (1982) Arch. Biochem. Biophys. **219**, 1–11
Carlier, M. F. & Pantaloni, D. (1973) Eur. J. Biochem. **37**, 341–354
Carlier, M. F. & Pantaloni, D. (1976*a*) Biochimie **58**, 27–35
Carlier, M. F. & Pantaloni, D. (1976*b*) Biochemistry **15**, 1761–1766
Carlier, M. F. & Pantaloni, D. (1978) Eur. J. Biochem. **74**, 553–563
Charles, A. M. (1970) Can. J. Biochem. **48**, 95–103
Colman, R. F. (1972) J. Biol. Chem. **247**, 215–223
Dalziel, K., McFerran, N., Matthews, B. & Reynolds, C. H. (1978) Biochem. J. **171**, 743–750
Dedhia, D., Meredith, M., Farina, R., Houston, M. & Shanler, A. (1979) Exp. Mycol. **3**, 229–239
Ehrlich, R. S. & Colman, R. F. (1976) Biochemistry **15**, 4034–4041
Farrell, H. M., Jr. (1980) Arch. Biochem. Biophys. **204**, 551–559
Fatania, H. R. & Dalziel, K. (1980) Biochim. Biophys. Acta **631**, 11–19
Führer, L., Kubicek, C. P. & Röhr, M. (1980) Can. J. Microbiol. **26**, 405–408
Habison, A., Kubicek, C. P. & Röhr, M. (1983) Biochem. J. **209**, 669–676
Howard, R. L. & Becker, R. R. (1970) J. Biol. Chem. **245**, 3186–3194
Ingebretsen, O. C. (1975) J. Bacteriol. **124**, 65–72
Kelly, J. M. & Hynes, M. J. (1982) J. Gen. Microbiol. **128**, 23–28
Kubicek, C. P. & Röhr, M. (1980) Biochim. Biophys. Acta **615**, 449–457
Kubicek, C. P. & Röhr, M. (1986) CRC Crit. Rev. Biotechnol. in the press
Kuchel, P. W., Reynolds, C. H. & Dalziel, K. (1980) Eur. J. Biochem. **110**, 465–473
Laemmli, U. (1970) Nature (London) **227**, 680–685
La Nauze, J. M. (1966) J. Gen. Microbiol. **44**, 73–81
Lowenstein, J. M. & Smith, S. R. (1962) Biochim. Biophys. Acta **56**, 385–387
Ma, H., Kubicek, C. P. & Röhr, M. (1981) FEMS Microbiol. Lett. **12**, 147–151
Mattey, M. (1977) FEMS Microbiol. Lett. **2**, 71–74
Meixner-Monori, B., Kubicek, C. P., Habison, A., Kubicek-Pranz, E. M. & Röhr, M. (1985) J. Bacteriol. **161**, 265–271
Morrisey, J. H. (1981) Anal. Biochem. **117**, 307–310
Niehaus, W. G. & Dilts, R. P. (1984) Arch. Biochem. Biophys. **228**, 113–119

Osmani, S. A. & Scrutton, M. C. (1983) Eur. J. Biochem. **133**, 551–560
Osmani, S. A. & Scrutton, M. C. (1985) Eur. J. Biochem. **147**, 119–128
Ramakrishnan, C. V. & Martin, S. M. (1955) Arch. Biochem. Biophys. **55**, 403–407
Ramakrishnan, C. V., Steel, R. & Lentz, C. P. (1955) Arch. Biochem. Biophys. **55**, 270–273
Reeves, H. C., Daumy, G. O., Lin, C. C. & Houston, M. (1972) Biochim. Biophys. Acta **258**, 27–39
Reynolds, C. H., Kuchel, P. W. & Dalziel, K. (1978) Biochem. J. **171**, 733–742
Röhr, M., Stadler, P. J., Salzbrunn, W. O. J. & Kubicek, C. P. (1979) Biotechnol. Lett. **1**, 281–286
Röhr, M., Kubicek, C. P. & Kominek, J. (1983) Biotechnology **3**, 419–454
Self, C. H., Parker, M. G. & Weitzman, P. D. J. (1973) Biochem. J. **132**, 215–221
Uhr, M. L., Thompson, V. W. & Cleland, W. W. (1974) J. Biol. Chem. **249**, 2920–2927

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