Two NAD^+-Isocitrate Dehydrogenase Forms in *Phycomyces blakesleeanus*. Induction in Response to Acetate Growth and Characterization, Kinetics, and Regulation of Both Enzyme Forms†

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ABSTRACT: Two forms of NAD^+-isocitrate dehydrogenase, named ICDH-1 and ICDH-2, have been identified and purified in *Phycomyces blakesleeanus* NRRL-1555(−). These enzyme forms may be separated by chromatography on DEAE-Sephacel. ICDH-2 induction was a response to the adaptation of *Phycomyces* growth on acetate as the carbon source. Both enzyme forms were octamers of 338 ± 30 kDa with apparently identical subunits of 40.5 ± 5 kDa, but they were distinguishable by their electrophoretic mobilities on polyacrylamide gel electrophoresis. Isoelectric pH values were 5.28 and 4.96 for ICDH-1 and ICDH-2, respectively. ICDH-2 was more stable to urea denaturation than ICDH-1. At pH 7.6, ICDH-1 showed a markedly sigmoidal kinetic behavior with respect to isocitrate. However, ICDH-1 and ICDH-2 showed hyperbolic kinetics with respect to NAD^+. The tribasic form of isocitrate (I^{3-}) and its magnesium complex (MI^-) are the true substrates for both enzyme forms. Kinetic data obtained with Mg^{2+} as a divalent cation for both enzyme forms are compatible with the kinetic mechanism proposed by Cohen and Colman (1974) [*Eur. J. Biochem. 47,* 35−45] but assuming some degree of interaction between binding sites for the active form of isocitrate. This report describes for the first time the existence of two forms of NAD^+-isocitrate dehydrogenase in filamentous fungi. From the changes in activity levels for each form, during the adaptation of *Phycomyces* to growth on acetate and taking into account the kinetic and regulatory properties of both enzyme forms, we discuss the role of ICDH-1 and ICDH-2 in the control of isocitrate flux in *Phycomyces*.

In microorganisms growing on acetate, isocitrate can be metabolized either by the citric acid cycle or by the glyoxylate bypass (Kornberg, 1966). In *Escherichia coli*, this branch point is regulated by reversible phosphorylation and inactivation of $NADP^+$-isocitrate dehydrogenase (Garnak & Reeves, 1979; LaPorte & Koshland, 1982; Nimmo, 1984; Koshland et al., 1985), but the regulation of isocitrate flux in eukaryotic microorganisms remains to be established. NAD^+-dependent isocitrate dehydrogenase [*threo*-D_s-isocitrate:NAD^+ oxidoreductase (decarboxylating), EC 1.1.1.41] is a complex allosteric enzyme, present in all eukaryotic cells, that catalyzes the oxidative decarboxylation of isocitrate to 2-oxoglutarate with concomitant reduction of NAD^+ [for review, see Colman (1983) and Chen and Gadal (1990)]. NAD^+-isocitrate dehydrogenase presumably plays an essential role in the citric acid cycle, as has been reported in *Saccharomyces cerevisiae* (Repetto & Tzagoloff, 1989; Keys & McAlister-Henn, 1990; Cupp & McAlister-Henn, 1991, 1992), whereas $NADP^+$-isoenzyme functions in separate metabolic pathways. *Phycomyces blakesleeanus* is a zygomycete fungus from which we have reported isocitrate lyase induction by acetate together with an *in vivo* catabolic inactivation by glucose of the derepressed enzyme (Rúa et al., 1989). Dedhia et al. (1979) had described the purification and characterization of $NADP^+$-isocitrate dehydrogenase from *P. blakesleeanus*. In the course of our investigations into the regulation of isocitrate flux in *Phycomyces*, we have detected the existence of two NAD^+-isocitrate dehydrogenase forms whose activities varied with the growth conditions. In this paper, we report for the first time the existence of two forms of NAD^+-isocitrate dehydrogenase in a unicellular and filamentous fungus. The potential physiological roles of both enzyme forms are discussed in light of the changes in activity levels for each enzyme form during the adaptation of *P. blakesleeanus* to growth on acetate and taking into account the kinetic and regulatory properties of both enzyme forms which we have purified to homogeneity and characterized. The results reported here support a control mechanism for the isocitrate flux in *Phycomyces* which is different from that reported in *E. coli* (LaPorte & Koshland, 1983; LaPorte et al., 1984; Walsh & Koshland, 1985) and in *S. cerevisiae* (Keys & McAlister-Henn, 1990; Cupp & McAlister-Henn, 1993).

EXPERIMENTAL PROCEDURES

Materials. *threo*-DL-Isocitrate (trisodium salt), *threo*-D_s-isocitrate (monopotassium salt), cycloheximide, AMP, glyoxylic acid (monohydrate), oxalacetic acid, ammonium persulfate, sodium dodecyl sulfate (SDS),[1] phenazine methosulfate, nitro blue tetrazolium, and Blue Dextran were purchased from the Sigma Chemical Co., St. Louis, MO. Phenyl-Sepharose 6 fast flow, Sephacryl S-300 superfine, DEAE-Sephacel, Blue Sepharose CL-6B, Sephadex G-25, Sephadex G-200, polybuffer exchanger PBE 94 and poly-

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buffer 74 for chromatofocusing, and M_r markers for SDS/PAGE were from Pharmacia Fine Chemicals, Uppsala, Sweden. NAD^+, NADH, EDTA, 1,4-dithiothreitol, PMSF, M_r markers for analytical gel filtration, acrylamide, and *N*,*N*′-methylenebisacrylamide were from Boehringer Mannheim, Mannheim, Germany. Bactocasitone was obtained from Difco, Detroit, MI. Lab-Lemco Powder was from Oxoid Ltd, London, U.K.; and agar Bios C was from Biolife, Milan, Italy. All other chemicals used were standard analytical grade and provided by Merck, Darmstadt, Germany.

NAD^+-Isocitrate Dehydrogenase Forms Purification. *P. blakesleeanus* wild-type strain NRRL-1555(−) was grown in a liquid minimal medium (Sutter, 1975) except that we used L-asparagine (2 g/L) as the nitrogen source and that 2% D-glucose was replaced when indicated by 2.73% acetate, as the only carbon source. The mycelium was harvested after 18 h of growth as described previously (Rúa et al., 1989). Dry weight determination was according to Alonso et al. (1988).

All the purification steps were performed at 0−4 °C. The mycelia mass of *P. blakesleeanus* collected by filtration was cut into pieces and suspended in buffer A [10 mM sodium phosphate buffer (pH 6.5) containing 0.5 mM KCl, 2 mM $MgCl_2$, 4 mM EDTA, 5 mM 2-mercaptoethanol, and 50 μM PMSF] at a ratio of 5 mL/g wet weight and homogenized in a Braun MSK homogenizer cell disruptor for two 35 s cycles with a 15 s interval. The homogenate was centrifuged at 20300*g* for 25 min. The supernatant was filtered (crude extract) and adjusted to 25% saturation with $(NH_4)_2SO_4$ by slow addition of solid $(NH_4)_2SO_4$. The material was stirred for 20 min in an ice−water bath and centrifuged at 15300*g* for 20 min, and the pellet was discarded. Further solid $(NH_4)_2SO_4$ was added to the supernatant to give 55% saturation, and the suspension was stirred and centrifuged as above. The pellet was dissolved in a small volume (equivalent to $^1/_{10}$ of the volume of crude extract) of buffer B [5 mM sodium phosphate buffer (pH 7.5) containing 4 mM EDTA, 5 mM sodium citrate, 2 mM $MgCl_2$, and 5 mM 2-mercaptoethanol]. Solid $(NH_4)_2SO_4$ was added to give 1.7 M $(NH_4)_2SO_4$, and the suspension was loaded (in 4−6 mL fractions) onto a column (1.5 cm × 22.0 cm) of Phenyl Sepharose 6 fast flow, equilibrated with buffer B containing 1.7 M $(NH_4)_2SO_4$. NAD^+-isocitrate dehydrogenase activity eluted at the end of a linear gradient of 1.7 to 0 M $(NH_4)_2SO_4$ in buffer B (total volume of 100 mL) at a flow rate of 30 mL/h. Active fractions with at least half of the maximum activity were pooled, and all this material was applied (in 10−15 mL fractions) onto a Sephacryl S-300 column (3 cm × 40 cm) equilibrated with buffer C [20 mM Tris/HCl buffer (pH 7.5) containing 4 mM EDTA, 5 mM sodium citrate, 2 mM $MgCl_2$, and 5 mM 2-mercaptoethanol]. Active fractions which eluted with the same buffer at a flow rate of 10 mL/h were pooled, and this material was chromatographed on a DEAE-Sephacel column (1.5 cm × 16 cm) equilibrated with buffer C. NAD^+-isocitrate dehydrogenase activity was resolved by this chromatography in two activity peaks, the first of which (ICDH-1 activity) was eluted with buffer C and the second one (ICDH-2 activity) was eluted at 0.5 M NaCl with a linear gradient of 0 to 1.0 M NaCl in buffer C (80 mL total volume) at a flow rate of 15 mL/h. From this last step, the purification procedures diverge as the mycelia was from cultures on glucose or on acetate, as the sole carbon source. ICDH-1- and ICDH-2-containing peaks from glucose cultures were further purified by Blue Sepharose CL-6B chromatography. Active fractions from both enzyme forms were applied in separate experiments (in 4−5 mL fraction) onto a Blue Sepharose CL-6B column (1.5 cm × 5 cm) equilibrated with buffer C. For both cases, the column was washed with the same buffer until the absorbance at 280 nm had returned to base line, and NAD^+-isocitrate dehydrogenase activity was eluted in 1 mL fractions at a flow rate of 15 mL/h at 0.55−0.6 M NaCl with a linear gradient of 0 to 2.0 M NaCl in buffer C. On the other hand, when the enzyme forms were from acetate cultures, only ICDH-2 was purified as described above by means of a Blue Sepharose CL-6B column. The ICDH-1-containing peak was desalted through a PD-10 Pharmacia disposable column equilibrated with buffer D [20 mM Tris/HCl (pH 7.5) containing 2 mM $MgCl_2$, 5 mM 2-mercaptoethanol, and 4 mM EDTA]. Active fractions were applied onto a DEAE-Sephacel column (1.5 cm × 16 cm) equilibrated with buffer D, and NAD^+-isocitrate dehydrogenase activity was eluted at 0.15−0.16 M NaCl with a linear gradient of 0 to 1.0 M NaCl in buffer D (80 mL total volume).

Analytical Gel Filtration. A column (3 cm × 27 cm) of Sephadex G-200 was equilibrated with buffer B. The markers used were ferritin (M_r = 450 000), catalase (M_r = 232 000), aldolase (M_r = 158 000), bovine serum albumin (M_r = 68 000), ovalbumin (M_r = 45 000), chymotrypsinogen A (M_r = 25 000), and cytochrome *c* (M_r = 12 500). Alternatively, we also used a column (2.5 cm × 35 cm) of Sephacryl S-300 equilibrated with the same buffer B using the markers cited above except cytochrome *c*, which was replaced with tyroglobulin (M_r = 669 000).

PAGE. PAGE under nondenaturing conditions was performed on gel slabs (4% acrylamide and 0.15% bisacrylamide at pH 9.5) by a modification of the method of Bryan and Davis as described in the Sigma *Technical Bulletin n° MKR-137* (1986) with a Bio-Rad Miniprotean II apparatus modified by using special spacers which allowed us to obtain a gel with a thickness of 1.5 mm. For protein stain, the silver stain method described by Heukeshoven and Dernick (1985) was used. For enzyme activity stain, we used the assay system described by Gabriel (1971). SDS/PAGE was carried out on gel slabs (Miniprotean II) with 10% acrylamide and 0.1% SDS in Tris/glycine/SDS buffer according to Laemmli's method (1970). The subunit M_r markers used were α-lactalbumin (M_r = 14 000), trypsin inhibitor (M_r = 20 100), carbonic anhydrase (M_r = 30 000), ovalbumin (M_r = 43 000), bovine serum albumin (M_r = 67 000), and phosphorylase *b* (M_r = 94 000). For protein stain, we used the method described by Weber and Osborn (1969).

Chromatofocusing. For isoelectric point determination, we used a column (1 cm × 14 cm) of polybuffer exchanger PBE 94 equilibrated with 0.025 M histidine/HCl buffer (pH 6.2). Samples of enzymatic preparations were loaded onto the column, and the elution was carried out with polybuffer 74/

[1] Abbreviations: EDTA, ethylenediaminetetraacetic acid; ICDH-1, NAD^+-isocitrate dehydrogenase form 1; ICDH-2, NAD^+-isocitrate dehydrogenase form 2; PMSF, phenylmethanesulfonyl fluoride; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate; C^{3-}, tribasic form of citrate; C_t, total citrate; HC^{2-}, dibasic form of citrate; HI^{2-}, dibasic form of isocitrate; I^{3-}, tribasic form of isocitrate; I_t, total isocitrate; M^{2+}, free metal; M_t, total metal; MHC, metal complex of dibasic citrate; MHI, metal complex of dibasic isocitrate; MI^-, metal complex of tribasic isocitrate.

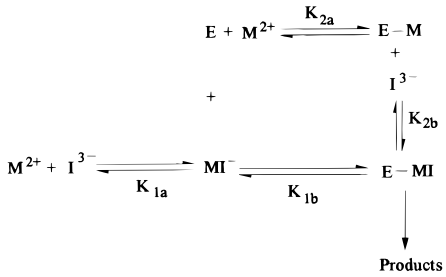
HCl (1/8 v/v, dilution factor) (pH 4.0) according to the Pharmacia Manual at a flow rate of 40 mL/h. The samples were desalted previously through Sephadex G-25 PD-10 disposable columns (Pharmacia).

NAD$^+$-Isocitrate Dehydrogenase Assay. NAD$^+$-isocitrate dehydrogenase activity was routinely assayed at 30 °C following the NAD$^+$ reduction by the absorbance change at 340 nm (ϵ = 4787.6 M^{-1} cm^{-1}). The standard assay contained, in 0.2 M Tris/acetate buffer (pH 7.6), 2 mM NAD$^+$, 3 mM *threo*-DL-isocitrate, 3 mM Mg^{2+} (as $MgCl_2$), and purified enzyme in a final volume of 1 mL. A unit of enzyme activity is defined as the amount of enzyme required to catalyze the formation of 1 μmol of NADH per minute under standard conditions. Specific activity refers to units per milligram of protein. Protein concentration was measured by the method of Lowry et al. (1951) or by the method of Warburg and Christian described by Layne (1957). Bovine serum albumin was used as a standard. The racemic mixture *threo*-DL-isocitrate was routinely used in the experiments, except in kinetic experiments, in which we used *threo*-D_s-isocitrate. In all kinetic experiments, the enzymatic preparations used of both enzyme forms were obtained as the eluates of DEAE-Sephacel chromatography.

Data Analysis. The distribution analyses of the species for the isocitrate and its metal complexes were carried out as described by Cohen and Colman (1972), taking into account that in the pH range tested total isocitrate (I_t) is primarily present in its dibasic (HI^{2-}) and tribasic (I^{3-}) forms and their corresponding metal complexes, MHI and MI^-. Total metal (M_t) is equal to the sum of free metal (M^{2+}) and the metal complexes of isocitrate. When the metal was Mg^{2+}, the stability constants used for MHI (defined as $[MHI]/[M^{2+}][HI^{2-}]$) and MI^- (defined as $[MI^-]/[M^{2+}][I^{3-}]$) complexes were 0.027 mM^{-1} (Grzybowski et al., 1970) and 1.35 mM^{-1} (Duggleby & Dennis, 1970) respectively, and the dissociation constant of dibasic isocitrate HI^{2-} (defined as $[H^+][I^{3-}]/[HI^{2-}]$) was 1.78 $\times$ 10^{-6} M (Grzybowski et al., 1970). When the metal was Mn^{2+}, the stability constant values used for MHI and MI^- were 0.057 and 1.15 mM^{-1}, respectively (Grzybowski et al., 1970). Since the association constants for the formation of the magnesium− or manganese−NAD$^+$ complexes were 106 M^{-1} (Duggleby & Dennis, 1970) and 78 M^{-1} (Cohen & Colman, 1972), respectively, these species constituted a negligible proportion of the added metal under the conditions used and were not considered.

The distribution analysis of the corresponding species of isocitrate when the experiments were carried out in the presence of citrate was calculated as follows. Since these experiments were carried out at pH 7.6, isocitrate and citrate should be almost fully ionized (i.e. $[I_t] \cong [I^{3-}]$ and $[C_t] \cong [C^{3-}]$), and thus, the formation of HI^{2-}, HC^{2-}, MHI, and MHC should be negligible. Thus, we include the stability constant value for the formation of Mg−citrate$^-$ complex of 1.78 mM^{-1} (Gabriel & Plaut, 1991). In each case, substitution of the appropriate constants into mass conservation equations results in a quadratic equation in which M^{2+} is the only unknown. A weighted nonlinear least-squares Marquardt regression of the initial velocity data was fitted to the Hill and Michaelis−Menten equations by means of the Enzfitter program (Leatherbarrow, 1987). The analysis of the role of metal in the kinetics of both forms of NAD$^+$-isocitrate dehydrogenase, ICDH-1 and ICDH-2, was made according to the kinetic model reported for the calf heart enzyme (Siliski & Colman, 1974) and for pig heart enzyme (Cohen & Colman, 1974) as indicated in Scheme 1. K_{1a},

Scheme 1



K_{1b}, K_{2a}, and K_{2b} are dissociation equilibrium constants. Initial velocity data were fit to Hill equations 1 and 2. $S_{0.5}$ for MI^-, $S_{0.5}$ for I^{3-}, and V'_{max} are described in terms of the intrinsic equilibrium constants of the model, according to the following equations:

$$v = V'_{max}/[1 + (S_{0.5}/[MI^-])^{n_H}] \quad (1)$$

$$v = V_{max}/[1 + (S_{0.5}/[I^{3-}])^{n_H}] \quad (2)$$

$$S_{0.5} \text{ for } MI^- = K_{1b}/(1 + K_{2b}/[I^{3-}]) \quad (3)$$

$$S_{0.5} \text{ for } MI^- = K_{1b}(1 + [M^{2+}]/K_{2a}) \quad (4)$$

$$S_{0.5} \text{ for } I^{3-} = K_{2b}(1 + K_{2a}/[M^{2+}]) \quad (5)$$

$$V'_{max} = V_{max}/(1 + K_{2b}/[I^{3-}]) \quad (6)$$

RESULTS

Time Course of Production of Two NAD$^+$-Isocitrate Dehydrogenase Forms from P. blakesleeanus. NAD$^+$-isocitrate dehydrogenase activity was found in crude extracts from mycelium of *P. blakesleeanus* grown in a liquid minimal medium with 2% glucose or 2.73% acetate as the carbon source. The enzyme activity from both cultures was maximal at about 18 h of growth, coincident with the onset of the exponential growth phase, decreasing quickly from this time. However, levels of enzyme activity, expressed as units per gram dry weight, were 2−3 times higher in the acetate-grown culture than on glucose. Isocitrate dehydrogenase variation in activity levels has also been reported in yeast (Polakis & Bartley, 1965; Machado et al., 1975), again with higher values on acetate than on glucose. Recently, furthermore, differential expression of the enzyme in response to yeast growth with different carbon sources has been described (Keys & McAlister-Henn, 1990; Haselbeck & McAlister-Henn, 1993), showing an increase in enzymatic protein when a culture grows on 3-carbon or 2-carbon nonfermentable metabolites with respect to the growth on glucose. Crude extracts from both cultures, previously desalted through Sephadex G-25 PD-10 disposable columns, were chromatographed on a DEAE-Sephacel column (1 cm $\times$ 8 cm) equilibrated with buffer B. NAD$^+$-isocitrate dehydrogenase activity was resolved in two activity peaks. The first peak, named ICDH-1, eluted with the equilibration buffer (buffer B), and the second activity peak, which eluted at 0.15 M NaCl when a linear gradient of 0 to 0.5 M NaCl in buffer B was applied, was named ICDH-2. With both

extracts, approximately 90% of the total units of NAD^+-isocitrate dehydrogenase activity applied to the column were recovered in the combined peaks of activity. ICDH-1 activity showed a sigmoidal dependence of velocity on isocitrate concentration, whereas ICDH-2 activity gave hyperbolic kinetics under the same experimental conditions. These results suggested the existence of two different forms of the NAD^+-isocitrate dehydrogenase in *P. blakesleeanus*. It is noticeable that two forms for NAD^+-isocitrate dehydrogenase have only been reported in the potato (*Solanum tuberosum*) (Laties, 1983; Tezuka & Laties, 1983). The time course profiles for both enzymatic activities, from glucose and acetate *Phycomyces* cultures, were similar, reaching the maximal activity at 18 h of growth. In crude extracts obtained from 18 h-old mycelium (3 g wet weight) of glucose cultures, the units of ICDH-1 were 7-fold higher than those of ICDH-2. In contrast, in crude extracts obtained from 18 h-old mycelium (3 g wet weight) of acetate cultures, the units of ICDH-1 were nearly half those of ICDH-2. While ICDH-1 levels were only moderately changed with the carbon source for growth, ICDH-2 levels from acetate cultures were at 18 h of growth 12-fold higher than those obtained from glucose ones and 50-fold higher at 24 h of growth. Thus, the *Phycomyces* adaptation to growth on acetate appears to be followed by an increase in the ICDH-2 activity.

Role of Glucose and Acetate in the Formation of NAD^+-Isocitrate Dehydrogenase Forms. Figure 1A illustrates the time course of both enzyme forms for an experiment of carbon source transfer from acetate to glucose. For ICDH-2, the profile is nearly the same and the activity lower than that of the control from transfer indicates or accounts for the increase in dry weight of the mycelium with glucose. With regard to ICDH-1, a stabilization after transfer to glucose may be seen, owing to a fast increase in ICDH-1 on glucose, which balances the increase in dry weight. Thus, it appears that there is no catabolic inactivation by glucose of ICDH-2 or by any other regulatory system of inactivation mediated through the growth on glucose.

Figure 1B shows the time course of both enzyme forms for an experiment of carbon source transfer from glucose to acetate. The absence of glucose noticeably slowed the relative activity decay of ICDH-1, but the addition of acetate brought it on. In the case of ICDH-2, the presence of acetate was noticeably necessary for a large increase in the relative activity. The adaptation to acetate is also accompanied by the synthesis of ICDH-1. The increase in the activity shown by both enzyme forms cannot be explained by a direct activation effect of acetate, since its concentration is lowered with the time of culture. Eighteen hours after the addition of acetate (60 h of culture), the onset of induction of synthesis of both enzyme forms was noticeable. The relative activity of ICDH-2, 12 h after this lag period, was 14-fold higher than the maximum value found on glucose cultures. The relative activity of ICDH-1 reached approximately 75% of its maximum on glucose cultures. The lag period may be considered as the time needed by the organism to adapt to the new carbon source. In addition, since the activity of neither enzyme form increased when glucose was removed from the culture medium, the above conclusion concerning the absence of catabolic repression is sustained. However, NAD^+-isocitrate dehydrogenase in *S. cerevisiae* is subject to catabolite repression (Keys & McAlister-Henn, 1990). We

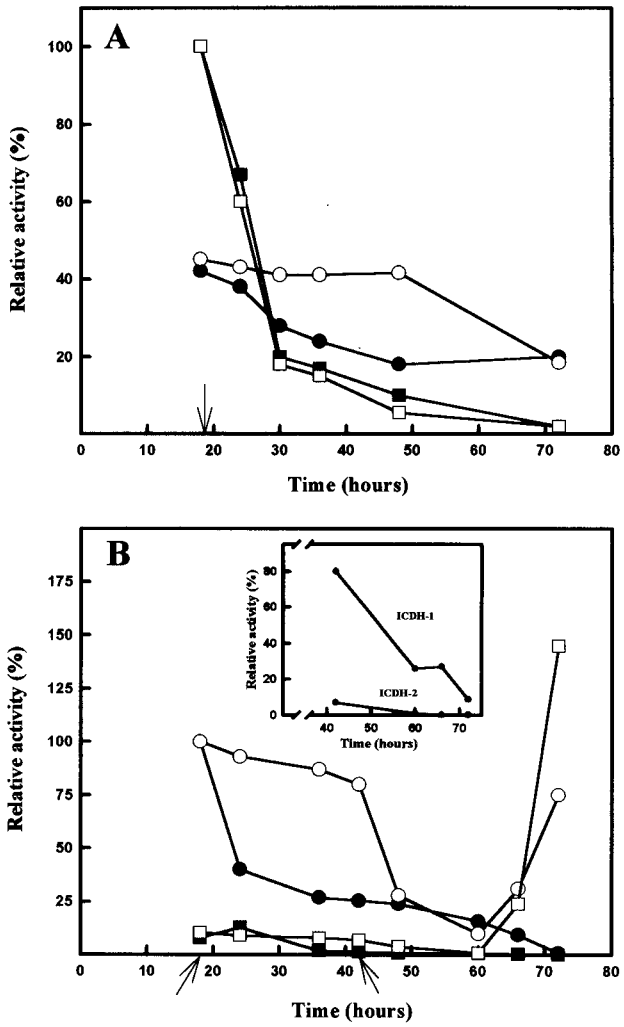


FIGURE 1: Time course of NAD^+-isocitrate dehydrogenase forms from mycelium of *P. blakesleeanus*. ICDH-1 and ICDH-2 activity were resolved by a DEAE-Sephacel column, as described in the text. (A) An 18 h-old *P. blakesleeanus* culture on 2.73% (w/v) acetate was exhaustively washed and immediately transferred to the same fresh defined medium with 2% (w/v) glucose as the sole carbon source. Activity values were obtained relative to the dry weight of mycelia and were expressed as percentages of the maximum activity found during growth on acetate for ICDH-2 (at 18 h). The arrow indicates the time of the transfer. Solid symbols represent the values obtained for ICDH-1 (●) and ICDH-2 (■) of a control culture grown from 0 to 72 h on acetate as the sole carbon source. Hollow symbols represent the values obtained for ICDH-1 (○) and ICDH-2 (□) after the transfer experiment. (B) An 18 h-old *P. blakesleeanus* culture on 2% (w/v) glucose was exhaustively washed and transferred to a fresh defined medium without a carbon source, and 2.73% (w/v) acetate was added 24 h after the transfer. Activity values were obtained relative to the dry weight of mycelia and were expressed as percentages of the maximum activity found during growth on glucose for ICDH-1 (at 18 h). Arrows indicate the time of transfer. Solid symbols represent the values obtained for ICDH-1 (●) and ICDH-2 (■) of a control culture grown from 0 to 72 h on glucose as the sole carbon source. Hollow symbols represent the values obtained for ICDH-1 (○) and ICDH-2 (□) after the transfer experiment. The inset shows the effect of 100 mg/mL cycloheximide added with acetate.

also tested the effect of cycloheximide (100 μg/mL) added along with acetate. No increase was detected in either case at 72 h of growth. These experiments are consistent with a *de novo* synthesis of both enzyme forms (Figure 1B, inset). Since levels of both enzyme forms decreased after the addition of cycloheximide, neither enzyme form appears to be the degradation product of the other. On the other hand,

Table 1: Summary of a Typical Purification Procedure of NAD$^+$-Isocitrate Dehydrogenase Forms from *P. blakesleeanus*[a]

step	volume (mL)	total activity (units)	total protein (mg)	specific activity (u/mg)	yield (%)	purification (-fold)
crude extract	47.0	73.62	447.52	0.16	100.0	1.0
$(NH_4)_2SO_4$ fractionation						
0−25% saturation	47.0	58.63	450.26	0.13	79.6	0.8
25−55% saturation	4.5	65.43	168.30	0.39	88.8	2.4
phenyl Sepharose eluate	14.0	33.65	30.74	1.09	45.7	6.8
Sephacryl S-300 eluate	17.5	18.27	6.56	2.80	24.8	17.5
DEAE-Sephacel eluate ICDH-1	10.5	3.73	0.62	6.02	5.1	37.6
DEAE-Sephacel eluate ICDH-2	5.8	5.45	0.41	13.30	7.4	83.0
DEAE-Sephacel eluate ICDH-1	23.5	3.00	0.08	37.50	4.1[b]	234.4[c]
Blue Sepharose eluate ICDH-2	9.5	4.40	0.09	48.90	6.0[b]	305.6[c]

[a] The crude extract was obtained from 10 g wet weight of 18 h-old mycelium of *P. blakesleeanus* grown on 2.73% acetate as the sole carbon source. For experimental details, see the text. [b] Yield and [c] purification factor for both enzyme forms are expressed relative to the total NAD$^+$-isocitrate dehydrogenase activity and the specific activity of the crude extract, respectively.

ICDH-2 samples from both acetate and glucose cultures, purified as set out below, were incubated (i) for 1 h with acid phosphatase grade I from potato (Boehringer) in 0.15 M sodium acetate buffer (pH 5) and (ii) for 1 h with alkaline phosphatase from calf intestine (Boheringer) in 0.2 M glycyne/NaOH buffer (pH 9.5). The samples were analyzed for enzyme activity, and we did not find any variation with regard to the control incubations. From these results, we consider that these NAD$^+$-isocitrate dehydrogenases are probably not regulated by a covalent modification reaction in contrast to that reported for bacterial NADP$^+$-isocitrate dehydrogenase (Garnak & Reeves, 1979; LaPorte et al., 1984).

Purification and Characterization of Two Forms of the NAD$^+$-Isocitrate Dehydrogenase (ICDH-1 and ICDH-2). The purification of ICDH-1 and ICDH-2 from mycelia of *P. blakesleeanus* grown on acetate as the sole carbon source resulted in preparations with specific activities of 37.5 and 48.9 units/mg of protein, respectively. Table 1 summarizes a typical purification procedure and data obtained for the two enzyme forms. When samples of ICDH-1 that had been separated from ICDH-2 were rechromatographed on the same DEAE-Sephacel column, only ICDH-1 was found and no ICDH-2 could be detected. This fact clearly indicates that ICDH-1 and ICDH-2 are two enzymatic forms and that their appearance was not a consequence of the breakdown of one form to the other. When citrate was removed from equilibration buffer C, ICDH-1 was retained on the DEAE-Sephacel column, owing to the decrease in the ionic strength of the buffer, and elution was achieved at 0.15−0.16 M of a linear gradient of NaCl with a 6-fold increase in the specific activity. Large losses of enzyme activity occurred during the first two chromatographic steps, although citrate was in the buffer to prevent enzyme inactivation (Barnes et al., 1971; Nealon & Cook, 1979), the enzyme activity probably being affected by dilution. The purification of ICDH-1 from mycelia of *Phycomyces* grown on glucose as the sole carbon source resulted in an enzymatic preparation with a similar specific activity. Both enzyme forms were very unstable; after 1 month at −20 °C in the presence of 5 mM sodium citrate, 2 mM EDTA, and 4 mM 2-mercaptoethanol, ICDH-1 and ICDH-2 respectively retained only 15 and 35% of their activity. However, the addition of 50% glycerol to the above mixture allowed ICDH-1 and ICDH-2 to retain, after 3 months, 60 and 70% of their activity, respectively. The homogeneity of the ICDH-1 and ICDH-2 final preparations was established by the existence of a single protein band in each case on PAGE (4% acrylamide), with a relative mobility of 0.21 and 0.3 for ICDH-1 and ICDH-2, respectively. Staining of the corresponding gels for NAD$^+$- isocitrate dehydrogenase activity (Figure 2) showed the enzymatic activity at the same position on the gel as the single protein band was located. A control gel, incubated in the tetrazolium assay mixture (Gabriel, 1971) without isocitrate, did not show the production of any formazan at the position of the protein band. Homogeneity of samples could also be seen by using PAGE in the presence of SDS, which yielded in both cases a single band with a relative mobility of 0.48 (Figure 2). From the position of this band relative to standard proteins, the subunit M_r was estimated to be 40 500 ± 5000. Gel filtration of both enzyme forms on a Sephacryl S-300 column gave an M_r of 338 000 ± 30 000. These data indicate that both enzyme forms are apparently composed of eight identical subunits. The octameric composition and the molecular mass determined fall in the range of other NAD$^+$-isocitrate dehydrogenases from several species [see for a review Chen and Gadal (1990)]. However, there is some discrepancy with regard to the identity of the subunits (Giorgio et al., 1970; Ramachandran & Colman, 1980; Keys & McAlister-Henn, 1990). The Stokes radii of both enzyme forms, calculated as described by Siegel and Monty (1966), were 6.1 nm. From this value, we have estimated for the diffusion coefficient $D_{20,\,W}$ a value of 3.52×10^{-7} cm^2 s^{-1}. These two values, obtained for both enzyme forms, are similar to those described for NAD$^+$-isocitrate dehydrogenase from pig's heart (Ehrlich et al., 1981). The respective isoelectric points were 5.28 and 4.96 for ICDH-1 and ICDH-2. Both ICDH-1 and ICDH-2 enzyme forms exhibited, for activity, an absolute requirement for divalent cations like Mg^{2+} or Mn^{2+} as has been reported for all NAD$^+$-isocitrate dehydrogenases studied (Colman, 1983; Chen & Gadal, 1990). When several metal ions were compared at 3.0 mM under the same conditions, the relative velocities for the oxidative decarboxylation reaction catalyzed by ICDH-1 or ICDH-2 were 100% for Mn^{2+}, 62−64% for Mg^{2+}, 34−42% for Co^{2+}, and 20−25% for Zn^{2+}. In addition, neither enzyme form showed activity when NADP$^+$ was substituted for NAD$^+$. The activity profiles as a function of pH were very similar for both enzyme forms with either Mg^{2+} or Mn^{2+} as a divalent cation, while the optimum pH values were broad and lay in the range of 7.5−9 in 0.2 M Tris/acetate buffer. A similar pH dependence has been reported for *Candida tropicalis* NAD$^+$-isocitrate dehydrogenase (Nabeshima et al., 1977). However, ICDH-1 and ICDH-2 from *P. blakesleeanus* showed optimum pH at more alkaline values than NAD$^+$-

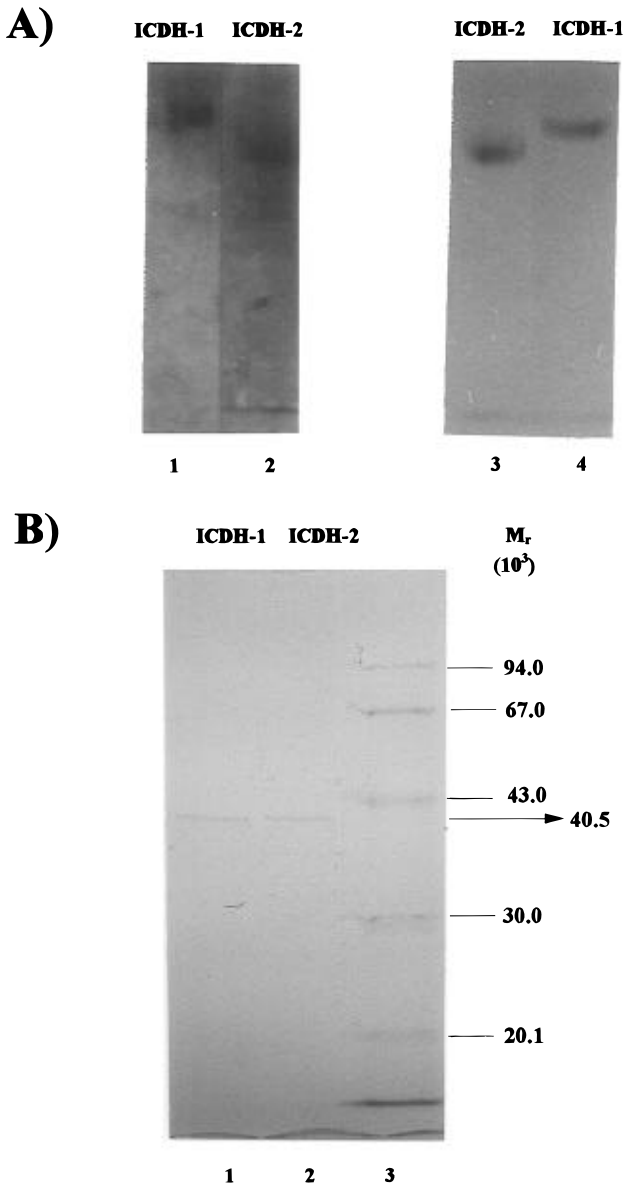


FIGURE 2: Polyacrylamide gel electrophoresis of NAD^+-isocitrate dehydrogenase forms from *P. blakesleeanus*. (A) Samples of purified ICDH-1 and ICDH-2 enzyme forms were electrophoresed on a nondenaturing polyacrylamide gel as described in Experimental Procedures. Samples for tracks 1 and 2 were stained for protein, and tracks 3 and 4 were stained for enzyme activity. (B) SDS/PAGE of the purified ICDH-1 and ICDH-2 enzyme forms as described in Experimental Procedures. Track 3 shows M_r markers (M_r values indicated at the right).

isocitrate dehydrogenase isoenzymes from *S. tuberosum* (Tezuka & Laties, 1983) or than the enzyme from other sources (Chen & Gadal, 1990). An Arrhenius plot of log $V_{max\ app}$ *versus* $1/T$ (data not shown) for both ICDH-1 and ICDH-2 gave similar nonlinear plots in the temperature range studied (25−48 °C). The apparent activation energy values at the lower temperatures for the two enzyme forms were 79 and 74.5 kJ mol^{-1} for ICDH-1 and ICDH-2, respectively. These values are similar to those reported for the NAD^+-isocitrate dehydrogenase from calf heart (Siliski & Colman, 1974) and from *Chlamydomonas reinhardtii* (Martínez-Rivas, 1991) in a similar temperature range (25−35 °C). The apparent activation energy values at the higher temperatures were 23.4 and 27.4 kJ mol^{-1} for ICDH-1 and ICDH-2, respectively, their respective transition temperatures being

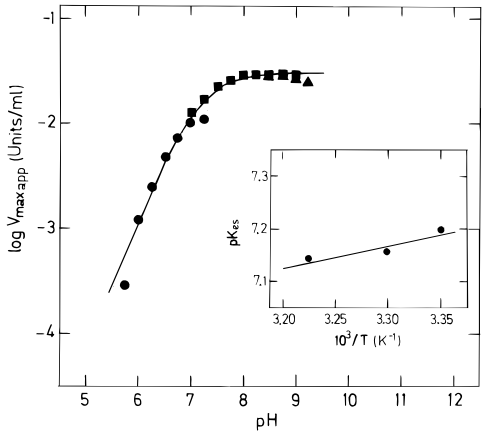


FIGURE 3: Variation with pH of log $V_{max\ app}$ for the oxidative decarboxylation of isocitrate in the presence of 0.75 mM free Mg^{2+}- at 30 °C catalyzed by the ICDH-1 enzyme form from *P. blakesleeanus*. The buffers used were 0.2 M citrate/phosphate (●), 0.2 M Tris/acetate (■), and 0.2 M glycine/NaOH (▲). The inset is the temperature dependence of the pK_{es} for the ionizable group on the acid side in the enzyme−substrate complex.

34 and 31 °C. These apparent activation energy parameters were irrespective of the divalent cation, Mn^{2+} or Mg^{2+}, used for activity. The presence of 0.3 mM citrate decreased 2−3-fold the apparent activation energy obtained at lower and at higher temperatures for both enzyme forms, without affecting their temperature dependency, which is consistent with an activator role for citrate.

The enzyme form ICDH-2 was more stable to urea treatment and to thermal inactivation than was ICDH-1. The inactivation process at urea concentrations of 3 and 4 M and 30 °C in buffer C followed first-order kinetics for both enzyme forms. Under these treatments, the half-life for ICDH-2 ranged between 493 and 15 min, while for ICDH-1, it ranged between 7 and 0.6 min at 3 and 4 M urea, respectively. ICDH-1 and ICDH-2 in buffer C and in the presence of bovine serum albumin (1 mg/mL) were inactivated by temperature, with first-order kinetics in the temperature range studied (30−60 °C). Variations in the enzyme concentration had no effect on the inactivation rate constants as expected for a first-order process (data not shown). From Arrhenius plots of inactivation rate constants (log k *versus* $1/T$ and log k/T *versus* $1/T$) (Segel, 1993), we have calculated the thermodynamic activation parameters. The values obtained were for enthalpy of 150 ± 7 and 177 ± 13 kJ mol^{-1} and for entropy of 0.16 ± 0.01 and 0.23 ± 0.02 kJ mol^{-1} K^{-1} for ICDH-1 and ICDH-2, respectively. Since $\Delta H^{\#}$ and $\Delta S^{\#}$ values for both enzyme forms were not significantly affected by temperature, thermal inactivation only resulted from an enhanced entropic contribution to $\Delta G^{\#}$, which in turn reflected the increased structural fluctuations of its polypeptide chains. This set of results indicates that ICDH-1 is more liable to suffer tertiary and quaternary disruption induced by either urea or heat than ICDH-2, as a consequence of different conformational states between them.

Effect of pH on the ICDH-1 and ICDH-2 Enzyme Forms and the Active Form of the Substrate. Figure 3 shows the pH dependence of the apparent V_{max} for the isocitrate oxidative decarboxylation catalyzed by ICDH-1. The apparent V_{max} profile has a limiting slope of +1 on the acid side. A similar V_{max} profile was obtained for ICDH-2. From this plot, we concluded that the apparent V_{max} was dependent

Table 2: Variation with pH of the Apparent $S_{0.5}$ for the Different Isocitrate Forms[a]

	$S_{0.5}$ (μM)									
	I_t		HI^{2-}		MHI		I^{3-}		MI^-	
pH	ICDH-1	ICDH-2	ICDH-1	ICDH-2	ICDH-1	ICDH-2	ICDH-1	ICDH-2	ICDH-1	ICDH-2
6.5	820	249	32.90	8.87	2.130	0.673	185	50	600	189
7.6	1355	363	5.00	1.08	0.290	0.080	355	76	1023	282
8.6	1700	659	0.67	0.21	0.030	0.014	480	150	1261	507
9.5	4893	1374	0.45	0.06	0.008	0.003	2565	370	2328	1000

[a] All reaction mixtures contained 2 mM NAD$^+$ and 3 mM Mg^{2+}, and the reaction was measured at 30 °C. The buffers used were 0.2 M imidazole/HCl at pH 6.5 and 0.2 M Tris/acetate at pH 7.6 and 8.6 and 0.2 M glycine/NaOH at pH 9.5.

on the basic form of an ionizable group of the enzyme−substrate complex. Fitting the experimental data to the following equation, $V_{max\ app} = V_{max}/(1 + [H^+]/K_{es})$, where K_{es} is the ionization constant for an activity-dependent amino acid residue on the enzyme−substrate complex, yielded a pK_{es} value of 7.15 for ICDH-1 and a pK_{es} value of 7.2 for ICDH-2. A sharp fall in activity from pH 9.5, probably reflecting an inactivation process, was observed. Additional information about the ionizable groups may be gained from examination of the temperature dependence of its pK. From the slope of a plot of pK_{es} *versus* $1/T$ (Figure 3 inset) according to the equation

$$pK_{es} = \Delta H°ion/2.3RT - \Delta S°/2.3R$$

the standard entalphy of ionization ($\Delta H°$ion) for the ionizable group in the enzyme−substrate complex was calculated to be 7.5 kJ mol^{-1} for ICDH-1. A similar value was obtained for ICDH-2. This $\Delta H°$ion value for the ionizable group on the acid side falls approximately in the range of carboxylic groups (Cohn & Edsall, 1943). The pK_{es} value is higher than that described for carboxylic groups (Cohn & Edsall, 1943), but a negatively charged as well as a hydrophobic environment can shift the pK value upward. When Mn^{2+} was replaced by Mg^{2+}, similar apparent V_{max} profiles were observed with both enzyme forms. The apparent V_{max} depends on the basic form of an ionizing group on the enzyme−substrate complex with a pK_{es} value of 6.8. The catalytic activity of the pig heart and the calf heart enzymes requires the nonprotonated form of an ionizing group of pK 6.3−6.6 which is independent of temperature (Ramachandran et al., 1974; Ramachandran & Colman, 1977; Siliski & Colman, 1974), suggesting the involvement of a carboxylic group. Hurley et al. (1990, 1991) have reported, for the *E. coli* NADP-isocitrate dehydrogenase, that in the Mg^{2+}-isocitrate binding site three aspartate side chains participate in the binding to Mg^{2+}. Alignment of the sequence of the *E. coli* enzyme and the IDH-2 subunit of *S. cerevisiae* NAD$^+$-isocitrate dehydrogenase indicates that a cluster of the residues which are on or near the binding site of isocitrate in the *E. coli* enzyme (Cupp & McAlister-Henn, 1991) is also conserved in IDH-2.

From the pH dependence of the $S_{0.5}$ for all four species of the isocitrate, we have determined which of these ionic species is the actual substrate for the ICDH-1 and ICDH-2 enzyme forms of *Phycomyces* NAD$^+$-isocitrate dehydrogenase. Table 2 shows the values obtained for the $S_{0.5}$ of those species in the pH range of 6.5−9.5. Similar results were obtained with both enzyme forms. With respect to total isocitrate, $S_{0.5}$ values increased about 6 times. A variation of approximately the same magnitude was detected for the I^{3-} and MI^- forms. In contrast, the $S_{0.5}$ values for both the HI^{2-} and MHI forms decreased between 70 and 150 times and 220 and 270 times, respectively, in the same pH range. These forms, dibasic isocitrate and its corresponding metal complex, cannot be the substrate for either *Phycomyces* ICDH-1 or ICDH-2 since the pH variation of their $S_{0.5}$ values must be explained by a pH effect on the affinity, which should be evident in the $S_{0.5}$ value for total isocitrate, which must decrease. On the other hand, an increase of 6 times in $S_{0.5}$ for total isocitrate did not account for the active isocitrate species being a protonated form. Therefore, we think that the tribasic form of isocitrate and its corresponding metal complex are the candidates for substrate, since the $S_{0.5}$ values for these species varied in accordance with that detected for total isocitrate.

The pH dependence of the $S_{0.5}$ values observed for total isocitrate, the tribasic form of isocitrate, and its Mg^{2+} complex can be described as follows:

$$S_{0.5\ app}/V_{max\ app} = S_{0.5}/V_{max} + S_{0.5}[H^+]/V_{max}K_e$$

where K_e is the ionization constant for an activity-dependent amino acid residue of the free enzyme. We obtained straight lines when $S_{0.5\ app}/V_{max\ app}$ was plotted *versus* $[H^+]$ for total isocitrate, the tribasic form of isocitrate, and its Mg^{2+} complex. From these plots, we obtained a similar value for pK_e of 6.3−5.9, which indicates that the overall negative charge of the substrates shifts the pK_{es} to higher values.

Kinetics, Role of Mg^{2+}, and Regulation by Effectors. We have studied the isocitrate oxidative decarboxylation catalyzed by ICDH-1 and ICDH-2 at pH 7.6 in 0.2 M Tris/acetate buffer and at 30 °C. The initial velocity data, expressed as a function of the concentration of both MI^- complex and I^{3-}, were obtained at fixed and different free Mg^{2+} concentrations and were fitted to eqs 1 and 2. The kinetic parameters are summarized in Table 3. A marked sigmoidal velocity dependence with the active substrate form is obtained for ICDH-1 (n_H value between 3.0 and 3.8), whereas for ICDH-2, the sigmoidal kinetics was less pronounced, with an n_H value between 1.4 and 2.0. The free Mg^{2+} concentration does not appear to alter significantly the n_H value or the apparent V_{max} for either enzyme form. A similar n_H value was obtained when the velocity curves were expressed in terms of total isocitrate, MI^- complex, and I^{3-} concentrations. This therefore allows us to rule out the possibility of sigmoidal kinetics being generated merely through a failure to express concentration in terms of the true substrate. The sigmoidal patterns probably revealed positive cooperative behavior between the binding sites of the substrate on ICDH-1 and on ICDH-2. Hill coefficient (n_H) values between 2.5 and 4 have been reported for NAD$^+$-isocitrate

Table 3: Kinetic Parameters for ICDH-1 and ICDH-2 as a Function of Free Mg^{2+} Concentration[a]

[free Mg^{2+}] (mM)	ICDH-1					ICDH-2				
		$S_{0.5}$ (mM)					$S_{0.5}$ (mM)			
	n_H	[MI^-]	[I_t]	[I^{3-}]	V_{max} (u/mg)	n_H	[MI^-]	[I_t]	[I^{3-}]	V_{max} (u/mg)
0.25	3.02 ± 0.13	0.360 ± 0.030	1.480 ± 0.095	1.060 ± 0.072	8.3 ± 0.6	1.60 ± 0.01	0.128 ± 0.001	0.532 ± 0.001	0.408 ± 0.068	15.0 ± 0.1
0.50	3.50 ± 0.15	0.337 ± 0.005	0.878 ± 0.001	0.541 ± 0.001	7.8 ± 0.1	1.42 ± 0.41	0.166 ± 0.092	0.430 ± 0.239	0.260 ± 0.140	16.0 ± 3.2
1.50	3.80 ± 0.01	0.418 ± 0.010	0.621 ± 0.001	0.213 ± 0.001	8.2 ± 0.1	1.40 ± 0.30	0.136 ± 0.030	0.210 ± 0.053	0.072 ± 0.018	13.6 ± 1.0
10.00	3.41 ± 0.06	0.581 ± 0.001	0.631 ± 0.010	0.051 ± 0.002	8.3 ± 0.2	1.40 ± 0.04	0.250 ± 0.020	0.281 ± 0.030	0.020 ± 0.002	16.0 ± 0.6
20.00	3.79 ± 0.39	0.851 ± 0.124	0.886 ± 0.087	0.035 ± 0.001	8.3 ± 0.6	1.92 ± 0.25	0.320 ± 0.030	0.333 ± 0.001	0.013 ± 0.001	14.6 ± 0.9
40.00	3.32 ± 0.35	1.682 ± 0.120	1.710 ± 0.023	0.031 ± 0.001	8.2 ± 0.3	2.00 ± 0.06	0.800 ± 0.025	0.820 ± 0.026	0.016 ± 0.002	15.0 ± 0.3

[a] Kinetic parameters for variable substrate were determined in 0.2 M Tris/acetate buffer at pH 7.6 at 30 °C and 2 mM NAD^+ as described in the text.

dehydrogenase from several sources (Atkinson et al., 1965; Duggebly & Dennis, 1970; Barratt & Cook, 1978; Tezuka & Laties, 1983; Cupp & McAlister-Henn, 1993). In addition, results of kinetic and ligand-binding studies indicating four binding sites per octamer for isocitrate have been reported (Kuehn et al., 1971; Ehrlich & Colman, 1990). The velocity curves as a function of the concentration of the complex MI^- are superposable up to 1.5 mM free Mg^{2+}, but at higher concentrations, velocity curves were superposable when expressed as a function of the concentration of I^{3-}. On the other hand, Table 4 shows the kinetic parameters for ICDH-1 and ICDH-2 at fixed and different free I^{3-} concentrations obtained from eq 1. The apparent V_{max} (V'_{max}) increases with free I^{3-} concentration to a constant value (V_{max}). Under these conditions, the $S_{0.5}$ value for MI^- complex could approach the K_{1b} value. These results suggested that the MI^- complex can be the actual substrate of ICDH-1 and ICDH-2 in the presence of low free Mg^{2+} concentrations, but when metal concentrations are high, the substrate binds as free tribasic isocitrate (I^{3-}) according to reaction Scheme 1. In addition, at high free Mg^{2+} concentrations, the divalent cation produces a competitive type inhibition with respect to the MI^- complex in both enzyme forms. However, no inhibition by free tribasic isocitrate (concentration range until 80 mM) was detected for ICDH-1 or for ICDH-2. Thus, these results rule out the possibility of the formation of enzyme−isocitrate complex and subsequent binding of metal. From plots of $S_{0.5}$ for MI^- *versus* [free Mg^{2+}] and of $S_{0.5}$ for I^{3-} *versus* 1/[free Mg^{2+}], according to eqs 4 and 5, we calculated K_{1b}, K_{2b}, and K_{2a} values for both ICDH-1 and ICDH-2. The V_{max} values were calculated from eq 6. Table 5 summarizes the kinetic constant values obtained. Since for both enzyme forms K_{1b} is lower than K_{2a} (ca. 30 and 60 times for ICDH-1 and ICDH-2, respectively), we concluded that the preferred pathway is the binding of the enzyme with the MI^- complex. K_{1b} equals $S_{0.5}$ for the MI^- complex when [free Mg^{2+}] $\ll$ K_{2a} or when [I^{3-}] $\gg$ K_{2b}, according to eqs 3 and 4. For *Neurospora crassa* NAD^+-isocitrate dehydrogenase (Barratt & Cook, 1978), results that qualitatively agree with Scheme 1 have been obtained.

The substitution of Mn^{2+} for Mg^{2+}, under the experimental conditions at which the MI^- complex should be the actual substrate, brings about a 6-fold decrease in the affinity of both enzyme forms for the MI^- complex. In addition, Mn^{2+} decreases the sigmoidal dependence of velocity *versus* the MI^- complex for ICDH-1 as shown by the fact that the Hill coefficient is lowered by a unit ($n_H = 2.7$), while it had no significant effect on the Hill coefficient for ICDH-2. A similar response has been reported for NAD^+-isocitrate dehydrogenase from *N. crassa* (Barratt & Cook, 1978) when Mn^{2+} was substituted for Mg^{2+}.

At 30 °C in 0.2 M Tris/acetate buffer at pH 7.6, a fixed 1.25 mM total Mg^{2+} or Mn^{2+} concentration, and a fixed 1.0 mM total *threo*-D_s-isocitrate concentration, both ICDH-1 and ICDH-2 showed a hyperbolic dependence of initial velocity on NAD^+ concentration. The affinity for NAD^+ (0.16−0.19 mM) of ICDH-2 was the same with either metal used, Mg^{2+} or Mn^{2+}. However, the affinity for NAD^+ of ICDH-1 was ca. 3 times lower with Mn^{2+} (0.33 mM) than with Mg^{2+}(1 mM). The NAD^+-isocitrate dehydrogenase from *Brassica napus* (Coultate & Dennis, 1969) and both NAD^+-isocitrate dehydrogenase isoenzymes from *S. tuberosum* (Tezuka &

Table 4: Kinetic Parameters for the ICDH-1 and ICDH-2 Enzyme Forms of *Phycomyces* as a Function of Free Isocitrate Tribasic Concentration[a]

		$S_{0.5}$ (mM)				
I^{3-} (mM)	n_H	[MI^-]	[Mg^{2+}_t]	[free Mg^{2+}]	V_{max} (u/mg)	
0.025	2.85 ± 0.01	0.440 ± 0.001	14.300 ± 1.410	13.801 ± 1.270	3.9 ± 0.4	
1.000	3.40 ± 0.42	0.311 ± 0.012	0.532 ± 0.051	0.230 ± 0.031	7.7 ± 0.5	ICDH-1
2.000	3.01 ± 0.08	0.281 ± 0.002	0.390 ± 0.000	0.110 ± 0.001	6.7 ± 0.1	
0.005	1.40 ± 0.08	0.042 ± 0.009	6.610 ± 0.261	6.500 ± 0.020	3.6 ± 0.3	
0.050	1.30 ± 0.05	0.160 ± 0.002	2.640 ± 0.010	2.530 ± 0.840	16.5 ± 0.8	ICDH-2
2.000	1.60 ± 0.01	0.110 ± 0.002	0.170 ± 0.020	0.050 ± 0.001	16.6 ± 0.1	

[a] Kinetic parameters for variable substrate were determined in 0.2 M Tris/acetate buffer at pH 7.6 at 30 °C and 2 mM NAD$^+$ as described in the text. Mg^{2+}_t was total magnesium.

Table 5: Kinetic Constants for ICDH-1 and ICDH-2 Enzyme Forms at pH 7.6 and 30 °C According to Reaction Scheme 1

	ICDH-1	ICDH-2
V_{max}[a] (s^{-1})	45 ± 7	84.4 ± 16
n_H	3.0−3.8	1.4−2.0
K_{1b} (mM)	0.316 ± 0.04	0.113 ± 0.03
K_{2b} (mM)	0.023 ± 0.001	0.014 ± 0.005
K_{2a} (mM)	10 ± 1	7.0 ± 0.7

[a] Taking a molecular mass value for each enzyme form of 338 kDa.

Laties, 1983) showed K_m values for NAD$^+$ close to the value reported here for ICDH-2, while a K_m value for NAD$^+$ similar to that obtained for ICDH-1 has also been reported for *N. crassa* NAD$^+$-isocitrate dehydrogenase (Barratt & Cook, 1978).

Figure 4 shows the effect produced by several effectors on each enzyme form, and the corresponding kinetic constants have been summarized in Table 6. Any of these effectors affected the V_{max} for each enzyme form. The addition of AMP to the reaction mixture caused a decrease in the $S_{0.5}$ for MI^- of ICDH-1, also giving a 2-unit decrease in the Hill coefficient value. AMP activation of NAD$^+$-isocitrate dehydrogenase has been reported from several microorganisms (Hathaway & Atkinson, 1963; Sanwal & Stachow, 1965; Nabeshima et al., 1977; Barratt & Cook, 1978; Gabriel & Plaut, 1986) with the common characteristic of the increase in isocitrate affinity not affecting V_{max}. However, the marked decrease in cooperativity shown by *Phycomyces* ICDH-1 contrasts with the insignificant decrease in the Hill coefficient reported for the yeast enzyme (Gabriel & Plaut, 1986; Cupp & McAlister-Henn, 1993). On the other hand, AMP produced no effect on the kinetic behavior of *Phycomyces* ICDH-2. Citrate has also been reported to be a modulator of most NAD$^+$-isocitrate dehydrogenases. Addition of citrate to the reaction mixture resulted in a 10-fold or a 5-fold decrease in the $S_{0.5}$ value for MI^- for ICDH-1 and ICDH-2, respectively (Figure 4), thus approaching these two values between them. For both enzyme forms, the $S_{0.5}$ value in terms of total isocitrate and of the tribasic form of isocitrate (I^{3-}) also decreased. The increased affinity for MI^- implies that the effect of citrate on the enzyme forms was the result of a direct interaction with a regulatory site on each enzyme form. The maximum responses observed with AMP plus citrate are consistent with the binding of these effectors to ICDH-1 on different regulatory binding sites, as has been reported for the NAD$^+$-isocitrate dehydrogenase from yeast (Gabriel & Plaut, 1991). However, for ICDH-2, the kinetic response to the presence of both AMP and citrate was essentially similar to the one

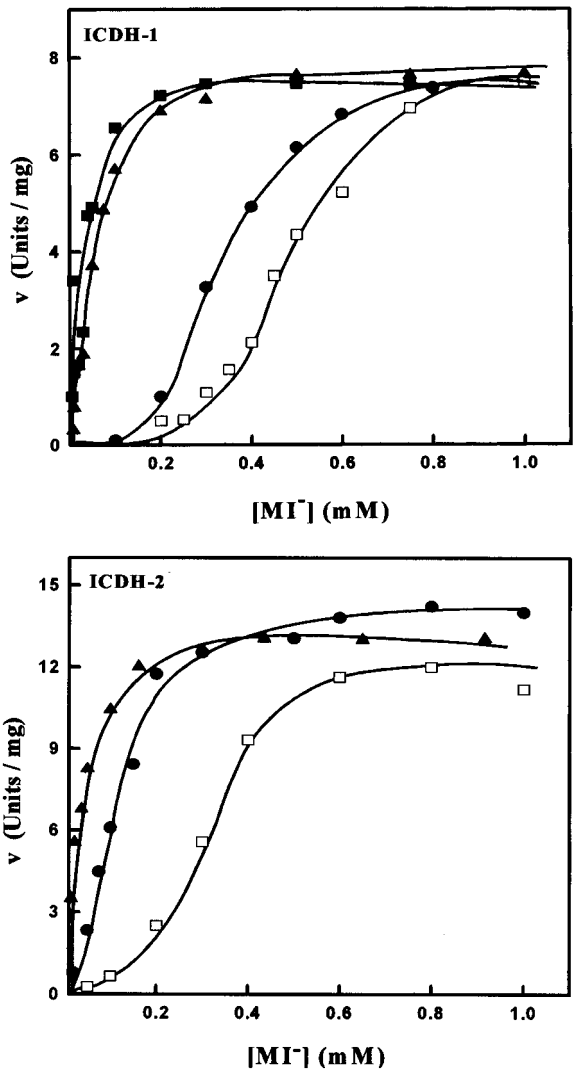


FIGURE 4: Effect of AMP, citrate, and NADH on the kinetic response of initial velocity with the magnesium−isocitrate complex (MI^-) concentration shown by the ICDH-1 and ICDH-2 enzyme forms of *Phycomyces*. The assays were carried out at 30 °C in 0.2 M Tris/acetate buffer at pH 7.6 at fixed 2 mM NAD$^+$ and fixed free Mg^{2+} concentrations of 0.5 and 0.25 mM for ICDH-1 and ICDH-2, respectively. No effectors (●), 0.5 mM AMP (■), 0.5 mM sodium citrate (▲), and 0.1 mM NADH (□).

observed with citrate alone, since this enzyme form appears to be desensitized to AMP.

In addition, 0.1 mM NADH produced a noticeable increase in the Hill coefficient and a slight increase in $S_{0.5}$ for the MI^- complex of ICDH-2, thus showing a kinetic behavior similar to that of ICDH-1 without any effector (Figure 4). However, the kinetic response of ICDH-1 was weaker, as

Table 6: Effect of AMP, Citrate, and NADH on ICDH-1 and ICDH-2 Kinetic Parameters for the MI^- Complex at pH 7.6[a]

	ICDH-1			ICDH-2		
	n_H	$S_{0.5MI^-}$ (mM)	V_{max} (u/mg)	n_H	$S_{0.5MI^-}$ (mM)	V_{max} (u/mg)
control	3.50 ± 0.15	0.337 ± 0.005	7.8 ± 0.1	1.60 ± 0.01	0.130 ± 0.006	15.0 ± 0.1
+citrate (0.5 mM)	2.55 ± 0.18	0.032 ± 0.001	7.4 ± 0.1	1.10 ± 0.10	0.030 ± 0.004	13.6 ± 0.7
+AMP (0.5 mM)	1.61 ± 0.08	0.054 ± 0.001	7.7 ± 0.1	1.75 ± 0.10	0.120 ± 0.008	15.6 ± 0.5
+citrate (0.5 mM) +AMP (0.5 mM)	1.61 ± 0.29	0.0254 ± 0.002	8.0 ± 0.2	1.00 ± 0.10	0.040 ± 0.005	16.5 ± 0.6
+NADH (0.1 mM)	4.00 ± 0.44	0.485 ± 0.019	8.0 ± 0.4	3.50 ± 0.03	0.300 ± 0.005	12.4 ± 0.1

[a] Kinetic parameters for the variable substrate, the complex of Mg^{2+} and the tribasic form of isocitrate were determined under the experimental conditions described in the legend to Figure 4.

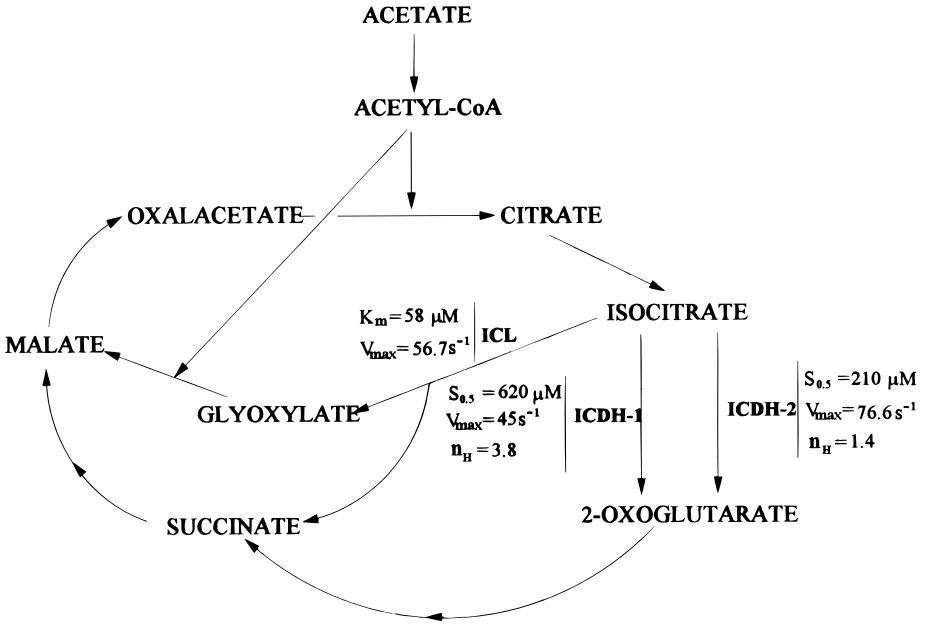


FIGURE 5: Branch point between isocitrate lyase and NAD^+-isocitrate dehydrogenase in *P. blakesleeanus*. Kinetic parameters for isocitrate lyase (pH 6.8) were reported by Rúa et al. (1990) and for NAD^+-isocitrate dehydrogenase forms ICDH-1 and ICDH-2 (pH 7.6 and fixed free 1.5 mM Mg^{2+} and 2 mM NAD^+) are provided in Table 4.

the Hill coefficient increased from 3.5 to 4.0 and the $S_{0.5}$ also increased slightly. So, the allosteric effector NADH exerts its effect mainly on ICDH-2. The different sigmoidal kinetics for both enzyme forms and the results obtained with these effectors may be explained assuming different interactions between subunits in each enzyme form, as a result of different conformational states. This is in agreement with the different physicochemical properties found for ICDH-1 and ICDH-2. The sigmoidal dependence of velocity *versus* isocitrate obtained for the NAD^+-isocitrate dehydrogenase from *N. crassa*, bovine heart, and the pea has been explained in terms of a mechanism which does not involve subunit interactions (Sanwal et al., 1965; Plaut et al., 1974; Duggleby & Dennis, 1970). However, recently (Cupp & McAlister-Henn, 1993), an allosteric model has been proposed for NAD^+-isocitrate dehydrogenase from yeast involving interactions between the regulatory (IDH-1) and the catalytic (IDH-2) subunits.

DISCUSSION

When *Phycomyces* grows on acetate as the sole carbon source, the glyoxylate pathway is essential for the production of cell constituents and thus *Phycomyces* induces isocitrate lyase (Rúa et al., 1989). In addition, *Phycomyces* also induces the ICDH-2 form of NAD^+-isocitrate dehydrogenase under this metabolic condition. The kinetic parameters (V_{max} and K_m for isocitrate) for isocitrate lyase previously reported by us (Rúa et al., 1990) together with the V_{max}, $S_{0.5}$, and n_H values for isocitrate reported here for both NAD^+-isocitrate dehydrogenase forms allow us to propose how the isocitrate flux is regulated in *Phycomyces* (Figure 5). The V_{max} value for isocitrate cleavage by isocitrate lyase is very close to the one obtained for ICDH-1, while the K_m value for total isocitrate for isocitrate lyase is lower than the $S_{0.5}$ value for ICDH-1. On the basis of the kinetic characteristics of ICDH-2, we think that *Phycomyces* induces this new enzyme form to participate in the regulation of the carbon flux through the Krebs cycle and the glyoxylate pathway. Kinetic analysis of both isocitrate dehydrogenase forms showed that ICDH-2 was a near-deregulated enzyme, following a kinetic behavior close to Michaelis−Menten. ICDH-2 shows a higher (ca. 2-fold) V_{max} value than ICDH-1 and a lower $S_{0.5}$ value than ICDH-1. In addition, the K_m value for NAD^+ of ICDH-2 is 6-fold lower than that of ICDH-1. While at an MI^- concentration equivalent to K_{1b} for ICDH-2, this enzyme form works at $0.5V_{max}$, and at the same MI^- concentration, the high Hill coefficient for ICDH-1 causes this enzyme form to work in a range of 0.04−0.02 of its V_{max} (Figure 6). In addition, the fact that V_{max} for ICDH-2 was about twice that for ICDH-1 confirms that ICDH-1 is not operative in a concentration range of MI^- close to the K_{1b} for ICDH-2. On the other hand, citrate decreased the $S_{0.5}$ value for

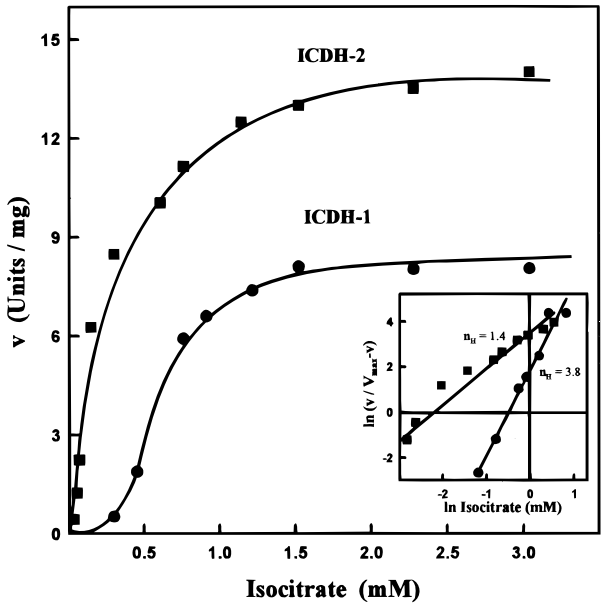


FIGURE 6: Initial velocity profiles for the isocitrate oxidative decarboxylation catalyzed by ICDH-1 and ICDH-2 enzyme forms, as a function of total *threo*-D_S-isocitrate concentration. The assays were carried out at 30 °C in 0.2 M Tris/acetate buffer at pH 7.6 at a fixed free Mg^{2+} concentration of 1.5 mM and 2 mM NAD^+. The inset is Hill plots of the data.

isocitrate for both ICDH-1 and ICDH-2 to a unique value similar to that calculated for K_m for isocitrate, for isocitrate lyase. Besides, the decrease of the Hill coefficient for isocitrate of ICDH-1 allowed this enzyme form to contribute to the total isocitrate flux through NAD$^+$-isocitrate dehydrogenase forms at ca. 30% at all isocitrate concentrations, in contrast with the absence of citrate. In addition, while NADH inhibits ICDH-2, approaching its behavior to ICDH-1, AMP activates ICDH-1 that now shows a behavior similar to that of ICDH-2. On the other hand, preliminary results showed that glyoxylate and oxalacetate produced a concerted inhibition on both enzyme forms, but this was weaker on ICDH-1 than on ICDH-2. At pH 7.6 in the presence of 2 mM NAD^+, 1.7 mM Mg^{2+}, and 2 mM *threo*-D_S-isocitrate, oxalacetate plus glyoxylate (5 mM each) produced a 17% inhibition of ICDH-1 but a 45% inhibition of ICDH-2. Glyoxylate alone had no inhibitory effect. The different kinetic and regulatory behaviors shown by the ICDH-1 and ICDH-2 enzyme forms are in agreement with the fact that each of them is responsible for the total NAD$^+$-isocitrate dehydrogenase activity in *Phycomyces* under different metabolic conditions.

On the other hand, during the transition generated by the transfer from acetate to glucose as the sole carbon source, (i) isocitrate lyase is rapidly inactivated by glucose (Rúa et al., 1989), (ii) citrate concentration falls, with the lowest value at 6 h after the transfer, as little as 50% of the original value (data not shown), and (iii) ICDH-1 retains full activity for 30 h after the transfer (Figure 1), thus allowing isocitrate to be metabolized.

The isocitrate flux through NAD$^+$-isocitrate dehydrogenase is lower when *Phycomyces* grows on glucose than when it grows on acetate, although the growth rate was between 1.5- and 2-fold higher on glucose than on acetate. This is consistent with an increase in the flux through the Krebs cycle on acetate cultures as reported for *E. coli* (Walsh & Koshland, 1985), and thus, the citrate concentration could be sufficiently high to produce the activation of both enzyme forms of the NAD$^+$-isocitrate dehydrogenase. So, it seems reasonable to conclude that *Phycomyces* growing on acetate reinforces the kinetic characteristics of ICDH-1 and induces ICDH-2 to compete with the isocitrate lyase, out of necessity induced to support growth. For the first time, it is reported that isocitrate flux through the Krebs cycle and glyoxylate pathways is regulated by the induction of an NAD$^+$-isocitrate dehydrogenase form. We cannot rule out the possibility of ICDH-1 and ICDH-2 being isozymes, and we are now carrying out experiments to clarify this point. The correlation of the enzyme forms with two distinct genes would be important for the regulatory model which we have described. This control mechanism is entirely different from that reported for *E. coli* (LaPorte & Koshland, 1983; LaPorte et al., 1984; Walsh & Koshland, 1985), with four-fifths of NADP$^+$-isocitrate dehydrogenase activity inactivated by phosphorylation.

In agreement with our results, the NAD$^+$-isocitrate dehydrogenase in *S. cerevisiae* is not apparently subject to any significant modification by phosphorylation and its specific activity increases substantially in acetate-grown cells (Keys & McAlister-Henn, 1990), but the control of isocitrate flux through the Krebs cycle appears to be exerted at least by the interaction between the regulatory subunit and the catalytic subunit of the proposed allosteric NAD$^+$-isocitrate dehydrogenase (Cupp & McAlister-Henn, 1993).

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