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PROPERTIES OF THE NICOTINAMIDE ADENINE DINUCLEOTIDE-SPECIFIC ISOCITRATE DEHYDROGENASE FROM *BLASTOCLADIELLA EMERSONII*

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SUMMARY

The regulatory properties of the NAD-specific isocitrate dehydrogenase (*threo*-D_S-isocitrate:NAD oxidoreductase (decarboxylating), EC 1.1.1.41) from *Blastocladiella emersonii* have been studied with steady state kinetic methods. The enzyme was found to catalyze the oxidative decarboxylation of isocitrate as well as the reversed reaction.

The results show that the enzyme is extremely sensitive to the buffer concentration in the assay mixture. This inhibition is reversed by the activators, AMP and citrate and is competitive with regard to isocitrate.

AMP reduces K_m for the substrates but has no effect on V. The enzyme exhibits positive homotropic cooperativity towards isocitrate both in the absence and presence of activators, while the cooperativity towards NAD disappears in the presence of activators as well as high isocitrate concentrations. The activation by AMP occurs according to normal Michaelis–Menten kinetics. AMP increases the pH optimum of the reaction.

The experimental results are consistent with the possibility that the enzyme contains two binding sites for isocitrate, a catalytic and a regulatory site. Binding of citrate as well as AMP mimics the effect of binding of isocitrate to the regulatory site.

INTRODUCTION

The aquatic non-filamentous Phycomycete *Blastocladiella emersonii* [1, 2] possesses two isocitrate dehydrogenases, one NAD and one NADP dependent [3–5]. The level of these enzymes changes considerably during differentiation, and it has been assumed that they play an important role in the metabolic changes occurring during development [1, 3, 4, 6].

The isolation and initial characterization of the NAD-specific isocitrate dehydrogenase (*threo*-D_S-isocitrate:NAD oxidoreductase (decarboxylating), EC

Abbreviation: HEPES, N-2-hydroxyethylpiperazine-N'-2-ethane sulfonic acid.

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1.1.1.41) from *Bl. emersonii* was first reported by LéJohn et al. [5]. Like the enzymes from *Neurospora crassa* [7] and yeast [8], the *Bl. emersonii* enzyme is activated by AMP and citrate. However, the present enzyme differed from the two other fungal enzymes in several ways [5]. Very high substrate concentrations were needed in the absence of AMP to obtain any activity at all. Moreover, it was found that the enzyme exhibited two distinct pH optima. Since baker's yeast and *N. crassa* belong to another class of fungi than *Bl. emersonii*, the isocitrate dehydrogenases from these organisms may differ considerably as previously found for their glutamate dehydrogenases [9–11].

The present study was carried out in order to characterize further the NAD-specific isocitrate dehydrogenase from *Bl. emersonii* and to compare the properties of this enzyme with those previously found for other isocitrate dehydrogenases.

MATERIALS AND METHODS

Materials

DL-Isocitrate (trisodium salt), α-ketoglutaric acid, NAD^+, NADH and all nucleotides used, as well as Tris and *N*-2-hydroxyethylpiperazine-*N'*-2-ethane sulfonic acid (HEPES) were obtained from Sigma Chemical Co., St. Louis, Mo. Whatman DEAE-cellulose DE-52 was purchased from Koch-Light Lab., Buckinghamshire, England, and Sephadex G-25 and G-200 from Pharmacia, Uppsala, Sweden.

Preparation of enzyme

The enzyme was purified from a single generation culture of *Bl. emersonii* [12] according to the method of LéJohn et al. [5] with some modifications.

The plants (100 g) were disrupted in a Potter–Elvehjem homogenizer. Cell debris were removed by centrifugation for 10 min at 35 000 × *g*. Solid $(NH_4)_2SO_4$ was added to the supernatant to reach stepwise 15, 30 and 55% satn. The 55% precipitate was collected and dissolved in 50 mM Tris–acetate buffer (pH 7.0) containing 10 mM K_2HPO_4, 1 mM $MgCl_2$, 0.1 mM Cleland's reagent and 3 mM citric acid (Buffer A). The protein solution was dialyzed for 3 h and loaded on a DEAE-cellulose column (2.5 cm × 30 cm). The protein was eluated with a linear gradient (0–0.5 M) of KCl made up in Buffer A. The enzyme appeared at about 0.25 M KCl. The fractions containing the enzyme were pooled and solid $(NH_4)_2SO_4$ was added to 65% satn. The precipitate was dissolved in a small volume of the same buffer as above, except that citric acid was omitted (Buffer B). The enzyme solution was passed through a Sephadex G-200 column (2.5 cm × 80 cm) and subsequently concentrated on a small DEAE-cellulose column (1.5 cm × 15 cm) (eluated with Buffer B containing 0.6 M KCl). The enzyme was desalted on a Sephadex G-25 column (1.5 cm × 15 cm) equilibrated with 0.1 M Tris–acetate buffer (pH 7.0), divided into small aliquots and frozen at −20 °C. The purified enzyme was stable for more than 3 months. The overall recovery of the enzyme was about 30%. The specific activity was 3–5 times higher than that obtained by LéJohn et al. [5]. All steps were performed at 1–3 °C.

Protein concentration was determined by the method of Lowry et al. [13]. Polyacrylamide-gel electrophoresis of the purified enzyme in the presence of sodium dodecylsulfate [14] revealed one major protein band.

Assay of enzyme activity

The activity was measured from the rate of increase in the absorption at 340

nm upon reduction of the coenzyme. A Gilford Model 2400 recording spectrophotometer was used. Unless otherwise indicated, the reaction mixture contained the following reagents in 30 mM Tris–acetate buffer (pH 7.5) (total volume 3 ml): 0.5 mM NAD^+, 3.3 mM isocitrate and 3.3 mM $MgCl_2$. The reaction was initiated by addition of enzyme (approx. 0.3 μg protein/ml). The pH was controlled at the end of the experiments. The measurements of the reductive carboxylation of α-ketoglutarate is described in the legend to Fig. 4. The temperature was in all experiments 25 °C.

RESULTS

In preliminary experiments it was found (Fig. 1A) that the activity of the NAD-dependent isocitrate dehydrogenase from *Bl. emersonii* is very sensitive to the Tris–

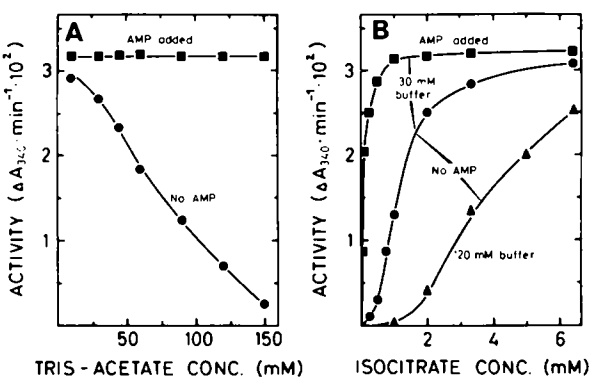

Fig. 1. Effect of buffer concentration on the activity of the NAD-dependent isocitrate dehydrogenase. (A) Enzyme activity in the absence and presence of AMP as a function of the Tris–acetate buffer concentration at pH 8.0. (B) Enzyme activity as a function of the isocitrate concentration in the presence of different Tris–acetate buffer concentrations at pH 7.5. The enzyme was assayed with 0.5 mM NAD^+, 3.3 mM $MgCl_2$ and, unless otherwise indicated, 3.3 mM isocitrate. AMP (1 mM) was added as indicated.

acetate buffer concentration when assayed in the absence of AMP. In the presence of the high buffer concentrations used by LéJohn et al. [5] (100–150 mM Tris–acetate buffer), the enzyme is nearly completely inhibited under the present conditions. The possibility should be considered that the high buffer concentration could be responsible for some of the unexpected results reported by LéJohn et al. [5].

The activity is unaffected by the buffer concentrations used when AMP is present. Citrate reversed the buffer inhibition in the same way as AMP. In the following experiments the buffer concentration was reduced to 30 mM Tris–acetate.

The results in Fig. 1B show the activity as a function of the isocitrate concentration. Although the same maximum velocity is obtained irrespectively of whether AMP is present or not, much higher isocitrate concentrations are needed to saturate the enzyme in the absence of AMP. With increasing buffer concentrations, the curves obtained in the absence of AMP are displaced more to the right, indicating that the inhibitory effect of the buffer is competitive with isocitrate.

In Fig. 2 is shown the effect of the NAD-concentration on the isocitrate satura-

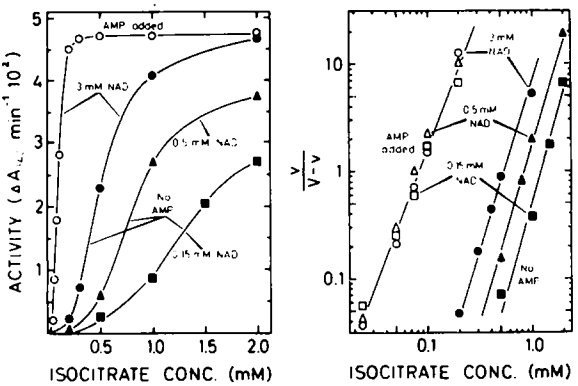

Fig. 2. Effect of isocitrate concentration on the activity of isocitrate dehydrogenase. The enzyme activity was determined as a function of the isocitrate concentration with different amounts of NAD^+ present. The activity was measured in 30 mM Tris-acetate buffer at pH 7.5 with 3.3 mM $MgCl_2$ and 1 mM AMP as indicated. Hill plots of the results are shown in the right panel.

tion curves. Sigmoid curves are obtained both in the absence and presence of AMP. When the results are plotted in Hill plot [15], straight lines with slopes, $n = 2.7$ were obtained. The maximum activities were the same in the absence and presence of AMP, for NAD^+ concentrations higher than about 0.2 mM. The isocitrate concentration giving half saturation ($S_{0.5}$) was independent of the NAD^+ concentration when the assay was carried out in the presence of AMP, but increased with decreasing NAD^+ concentrations in the absence of AMP.

The effect of the isocitrate concentration on the saturation curves for NAD^+ is demonstrated in Fig. 3. At low isocitrate concentrations, sigmoid curves were obtained in the absence of AMP while at high isocitrate concentrations, and in the presence of AMP normal hyperbolic curves were found. In Hill plots the slopes for the data obtained in the absence of AMP decreased from $n = 2.0$ for an isocitrate concentration of 0.25 mM to $n = 1$ at 3 mM isocitrate. Concurrently with the decrease in

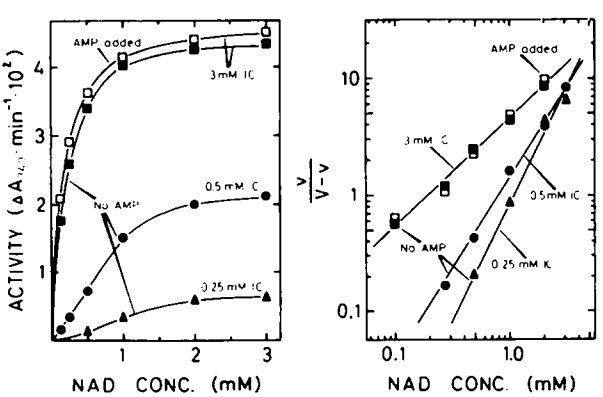

Fig. 3. Effect of NAD^+ concentrations on the activity of isocitrate dehydrogenase. The enzyme activity was determined as a function of the NAD^+ concentration with different amounts of isocitrate present. The activity was measured in 30 mM Tris-acetate buffer (pH 7.5) with 3.3 mM $MgCl_2$ and 1 mM AMP as indicated. Hill plot of the results is shown in the right panel.

TABLE I

EFFECT OF DIFFERENT NUCLEOTIDES AND CITRATE ON THE CATALYTIC ACTIVITY

The enzyme was assayed in 30 mM Tris–acetate buffer (pH 7.5) with 0.25 mM isocitrate, 0.5 mM NAD^+, 3.3 mM $MgCl_2$ and increasing concentrations of nucleotide. The activity in the presence of 1 mM 5′-AMP was set equal to 100%. K_A was determined from a Lineweaver–Burk plot [28]. In cases where K_A is not given, the added compound was present in a concentration of 1 mM.

Additions	Activity (%)	K_A (mM)
No	8	
5′-AMP	100	0.025
3′,5′-cyclic AMP	39	0.75
2′,3′-cyclic AMP	19	1.00
2′-AMP	5	
3′-AMP	30	1.00
5′-dAMP	95	0.12
5′-ADP	73	0.15
5′-ATP	6	
5′-GMP	6	
5′-GTP	8	
5′-CMP	6	
5′-UMP	8	
5′-UTP	8	
5′-TMP	4	
Citrate	100	0.05

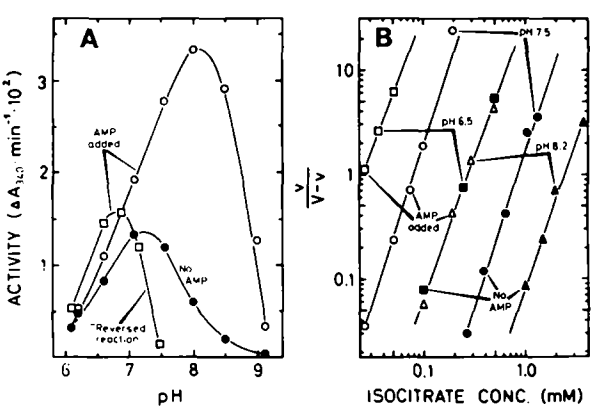

Fig. 4. Effect of pH on the AMP activation of isocitrate dehydrogenase. (A) Oxidative decarboxylation of isocitrate and reductive carboxylation of α-ketoglutarate as a function of pH. The oxidative decarboxylation was measured both in the absence and presence of 1 mM AMP. The assay mixture contained 1 mM isocitrate, 0.5 mM NAD^+ and 3.3 mM $MgCl_2$ in 30 mM Tris–acetate buffer. The reductive carboxylation of α-ketoglutarate was assayed with 30 mM α-ketoglutarate, 30 mM $NaHCO_3$, 3.3 mM $MgCl_2$ and 0.17 mM NADH, dissolved in 130 mM HEPES buffer containing 1.0 mM AMP. (B) Hill plot of the saturation curves for isocitrate measured in the presence and absence of AMP at different pH. The activity was measured at the pH indicated in the presence of 0.5 mM NAD^+ and 3.3 mM $MgCl_2$.

slope, the $S_{0.5}$ decreased. The Hill plots of the data found in the presence of AMP were all identical to that obtained for 3 mM isocitrate.

The activation of the enzyme by different nucleotides is shown in Table I. The activation occurs according to normal Michaelis–Menten kinetics. AMP, dAMP and ADP were the most efficient activators. Although these compounds activated the enzyme to nearly the same extent, K_{AMP} was only 1/5 of the corresponding constants for dAMP and ADP. The other adenosine phosphates were either unable to activate the enzyme or had only a moderate activating effect. Other nucleoside monophosphates had no effect on the enzyme activity. Some of the triphosphates seem to be slightly inhibitory. Citrate activated the enzyme to the same extent as AMP and was nearly equally effective on a molar basis. K_A for the activators was found to depend strongly on the isocitrate concentration. Thus, for instance, K_{AMP} decreased by a factor of 5 when the isocitrate concentration was doubled.

The enzyme had a pH optimum at about pH 8 when assayed in the presence of AMP (Fig. 4A). Under the present assay conditions the pH optimum decreased by about 1 unit when AMP was omitted from the assay mixture. In contrast to what was reported by LéJohn et al. [5], it was found that the enzyme is able to catalyze the reversed reaction, the reductive carboxylation of α-ketoglutarate to isocitrate. The pH optimum of the reversed reaction measured in the presence of AMP, occurred at pH 6.8. On a molar basis the enzyme catalyzed the oxidative decarboxylation of isocitrate approx. 50 times as efficient as it catalyzed the reversed reaction when measured at the respective pH optima. No carboxylation of α-ketoglutarate was observed in the absence of AMP. Interestingly, AMP reduced $S_{0.5}$ for isocitrate relatively to the same extent at all pH values studies (Fig. 4B). Moreover, the cooperativity is retained also at low pH.

DISCUSSION

The present results differ in several important respects from those previously reported by LéJohn et al. [5]. The discrepancies are mainly due to the high buffer concentrations used by the previous authors in spite of the fact that the enzyme is highly sensitive to the ionic strength. The present paper demonstrates that when the NAD-dependent isocitrate dehydrogenase from *Bl. emersonii* is assayed at low buffer concentrations, the half saturation values for the ligands decrease to the same order of magnitude as found for other NAD-dependent isocitrate dehydrogenases [16]. Moreover, we have been unable to observe more than one pH optimum. In contrast to LéJohn et al. [5], we found that the enzyme from *Bl. emersonii* catalyzed the reductive carboxylation of α-ketoglutarate with a similar efficiency as does the yeast enzyme [17].

It seems to be a general feature of the NAD-dependent isocitrate dehydrogenases that they have a greater affinity for isocitrate at lower pH values [18–21]. This is also reflected by the fact that at a given isocitrate level the effect of the activating nucleotides decreases with decreasing pH. The effect of pH on the homotropic cooperativity for isocitrate differs, however, for different enzymes. The enzymes from *Bl. emersonii* and yeast [21] show positive cooperativity for isocitrate both at pH 6.5 and 7.5. The enzymes from *N. crassa* [18] and Ehrlich ascites cells [22] show positive homotropic cooperativity at pH 7.5 while normal Michaelis–Menten saturation curves

are obtained at pH 6.5. On the other hand, no cooperativity is found for isocitrate with the enzymes from bovine heart [19] and hog liver [23].

Studies on the effect of different nucleotides and metabolites show that the enzyme from *Bl. emersonii* is fairly specifically activated by AMP and citrate. In contrast to the yeast enzyme [24], the activation of the present enzyme occurs according to normal Michaelis–Menten kinetics. Of the other nucleotides tested, it was only dAMP and ADP which activated the enzyme to nearly the same extent as AMP. However, with these nucleotides 5–6 times higher concentrations were needed. In previous studies it has been found that the effect of nucleotides on the NAD-specific isocitrate dehydrogenases varies with the source of the enzyme. Thus, some enzymes from bacteria and higher plants are not influenced at all by nucleotides [16, 25, 26]. The enzyme from yeast [24] and *N. crassa* [7] is activated specifically by AMP, while it is found that ADP is the most efficient activator of the enzyme from hog liver [23].

The observation that citrate mimics the effect of high isocitrate concentrations, suggest that the enzyme has two binding sites for isocitrate, a regulatory site and a catalytic site. Binding of isocitrate to the regulatory site reverses the inhibitory effect of high buffer concentration in a similar manner as does AMP and citrate. The fact that K_A for citrate is very small suggests that citrate can provide a precursor activation under physiological conditions in a similar way as has previously been suggested for the enzyme from *N. crassa* [27].

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