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α-KETOGLUTARATE DEHYDROGENASE FROM CAULIFLOWER MITOCHONDRIA: PREPARATION AND REACTIVITY WITH SUBSTRATES

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Key Word Index—*Brassica oleracea*; Cruciferae; mitochondria; α-ketoglutarate.

Abstract—The α-ketoglutarate dehydrogenase enzyme system (KGDH) was partially purified from mitochondria of cauliflower (*Brassica oleracea*) florets. After sonication of the mitochondria in the presence of amphotericin B, KGDH was separated by differential centrifugation in buffered sucrose solutions to give a final activity of 1–2 μmol/min/mg protein under standard assay conditions at 22°. The preparations had low levels of pyruvate dehydrogenase. Occasionally, preparations contained small amounts of the internal rotenone-insensitive NADH oxidase activity. The KGDH was fully active without added lipoamide dehydrogenase and showed optimal activity at pH 7.4. Analysis of the kinetics at fixed cofactor concentrations and variable α-ketoglutarate (KG) concentrations indicated that the V_{max} was three-fold higher at pH 7.4 than at pH 7.0. The apparent K_m for KG was 116 μM at pH 7.0 and 564 μM at pH 7.4, and there was no cooperativity with KG binding at either pH. The apparent K_m for NAD was 441 μM at pH 7.0 and 981 μM at pH 7.4. The apparent K_m for CoA was 15.7 μM at pH 7.0 and 12.2 μM at pH 7.4. The apparent K_m for thiamine pyrophosphate (TPP) was 106 μM at pH 7.0 and 87.5 at pH 7.4. Analysis of the n_{app} in response to varying concentrations of the co-factors (NAD, TPP, CoA) showed no cooperativity at pH 7.0 and a slightly positive cooperativity (1.2 to 1.5) at pH 7.4. The procedure provides a method for the rapid isolation of the intact cauliflower KGDH.

INTRODUCTION

The regulation of α-ketoglutarate (KG) utilization by α-ketoglutarate dehydrogenase (KGDH) is an essential aspect in the control of energy metabolism and carbon flow for the biosynthesis of glutamate, proline and arginine. KGDH catalyses the reaction of KG with CoA and NAD to produce carbon dioxide, succinyl-CoA, and NADH. α-Ketoglutarate dehydrogenase is a multienzyme complex with three activities: a thiamine pyrophosphate (TPP) dependent decarboxylating dehydrogenase, a lipoamide dependent transacylase and a lipoamide dehydrogenase (Lip-DH).

Plant mitochondria have low concentrations of endogenous co-factors, but under certain conditions can accumulate large amounts of NAD [1, 2], TPP [3], and CoA [4] suggesting a strong role for these co-factors in the regulation of mitochondrial matrix enzymes. NAD and other nucleotides are considered to be regulators of mitochondrial respiration [4]. The absolute requirement of KGDH for TPP [1], the active uptake of TPP and CoA by isolated mitochondria [4, 5] and the sharing of CoA and TPP by KGDH, pyruvate dehydrogenase (PDH) and some other mitochondrial dehydrogenases [6, 7] mean that the KGDH activity must be modulated by the varying mitochondrial concentrations of these co-factors and co-substrates. This situation necessitates the evaluation of the KGDH activity in the presence of varying amounts of these co-factors and co-substrates using an intact KGDH preparation.

Earlier kinetic studies on the cauliflower KGDH by Poulsen and Wedding [8] were performed on purified preparations of a salt-extracted, re-associated enzyme; the endogenous Lip-DH and enzyme-bound TPP were dissociated from the complex during this purification. In order to obtain activity with these preparations, pig heart Lip-DH had to be added to the assay mixture [8].

In this study, we report the partial purification of an intact KGDH preparation that was free from other competing KG utilizing activities and fully active in the absence of added Lip-DH.

RESULTS AND DISCUSSION

The method used to prepare the enzyme yielded about 25 μg protein/g of cauliflower, with a KGDH specific activity of 1 to 2 units/mg protein. The amount of PDH obtained in the preparation (20% of the KGDH activity) was comparable to that obtained by Poulsen and Wedding [8]. More than 50% of this PDH activity was lost upon storage at 8° for 24 hr.

Addition of amphotericin B to the mitochondria prior to sonication resulted in an increased yield of KGDH activity and a decreased NADH oxidase activity. This occasional NADH oxidase contamination, up to 10% of the total KGDH activity in some preparations, could not

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be separated from the KGDH either by elution on a cellulose–phosphate column or by centrifugation in a 30–60% sucrose density gradient. Treatment with hydroxylapatite resulted in the loss of both activities. The oxidase activity was more resistant to successive freeze-thaw and heating cycles (2 min at 90°) than KGDH activity. In addition, the oxidase was not inhibited by potassium cyanide, rotenone, antimycin A, amytal, and salicyl hydroxamate (SHAM). On the other hand, these compounds caused complete inhibition of the KGDH activity. In contrast to the PDH from molluscs [9], the cauliflower PDH activity was not inhibited by high concentrations of EGTA (5 mM) at pH 7.0 or 7.4. Addition of 2 mM EGTA did not inhibit the cauliflower KGDH activity at either pH 7.0 or 7.4.

Kinetics

Using the standard assay system with 50 mM Tris–acetic acid between pH 6.6 and 7.8 and in the presence of 250 μM KG and 1 mM EGTA, KGDH showed optimal activity at pH 7.4. Poulsen and Wedding [8] and Wedding and Black [10] found an optimal KGDH activity at pH 6.9 and 7.0, respectively.

The KGDH activity showed rectangular hyperbolic kinetic responses at pH 7.0 and pH 7.4 when velocities were plotted against KG concentrations. Analyses of the Hill plots at variable KG concentrations showed no cooperative effects (n_{app} of 1.0) at pH 7.0 and 7.4 and were in agreement with the previous results of Wedding and Black [10] at pH 7.0.

The apparent V_{max} with saturating KG concentrations at pH 7.4 was about 3.4-fold higher than that observed at pH 7.0. The apparent K_m for KG at pH 7.4 (564 μm) was higher than that found at pH 7.0 (116 μm). The latter K_m value was in agreement with the value of 120 μm observed by Poulsen and Wedding at pH 6.9 [8] but differed somewhat from the values of 260–270 μM reported by Wedding and Black [10] and Craig and Wedding [11].

At pH 7.0, the apparent K_m values for NAD, CoA and TPP were about 441, 15 and 106 μM, respectively, whereas at pH 7.4, these values were 981, 12 and 88 μM, respectively (Table 1). The apparent K_m values for NAD, CoA and TPP were three-fold higher than those of KGDHs from animal sources (see [12]). The K_ms for CoA and TPP were three-fold and 20-fold greater than the respective apparent K_ms for CoA and TPP reported by Poulsen and Wedding [8].

Table 1. Effect of NAD, CoA, and TPP on the kinetics of KGDH at pH 7.0 and 7.4

Variable* substrate	pH	K_m (μM)	V_{max} (units)	n_{app}
KG	7.0	116±21	29±2	0.94
KG	7.4	564±64	97±5	1.13
NAD	7.0	441±105	61±6	1.17
NAD	7.4	981±121	36±2	1.10
CoA	7.0	16±5	23±2	1.50
CoA	7.4	12±2	31±1	1.14
TPP	7.0	106±37	20±2	1.75
TPP	7.4	87±17	35±2	0.96

*Assays were performed at 22° in 1 ml reaction mixtures containing 50 μg of enzyme and 50 mM Tris–acetic acid, 3 mM DTT and 1 mM EGTA at the indicated pHs. When not the variable substrate, NAD, KG, CoA and TPP were held at the following respective concentrations: 1.5 mM, 0.25 mM, 0.3 mM and 0.3 mM. The reaction was initiated by the addition of the variable substrate to an otherwise complete reaction mixture.

The small differences between the apparent K_m values for CoA and KG at pH 7.0 (Table 1) and those observed by Poulsen and Wedding [8] at the same pH may be related to differences in the preparation of the enzyme or the source of tissue (see also [1, 3]). The differences in TPP binding probably result from the use of a Mg-TPP complex as the TPP substrate in the studies by Wedding [8, 10]. The K_m values reported for pH 7.4 (Table 1) probably reflect a more realistic picture of the *in vivo* physiological situation becuase plant mitochondrial pH is between 7.3 and 7.5 [13, 14]. The n values above 1 for co-factor binding at both pHs indicates some degree of cooperativity of co-factor binding in this enzyme complex.

NADH oxidase activity

The insensitivity of the NADH oxidase activity to rotenone, EGTA and Ca^{2+}, indicated that this enzyme is the internal rotenone-resistant, low affinity NADH oxidase [15] that is linked to the respiratory chain via the ubiquinone pool [16, 17]. This NADH oxidase has an apparent K_m for NADH about 84 μM [15, 18]. Therefore, at the very low concentrations of NADH produced during the assay of the KGDH reaction, a small amount of oxidase would not compromise the measurement of the KGDH activity. The values reported here for the KGDH activity (Table 1, Fig. 1) were obtained with NADH oxidase free preparations.

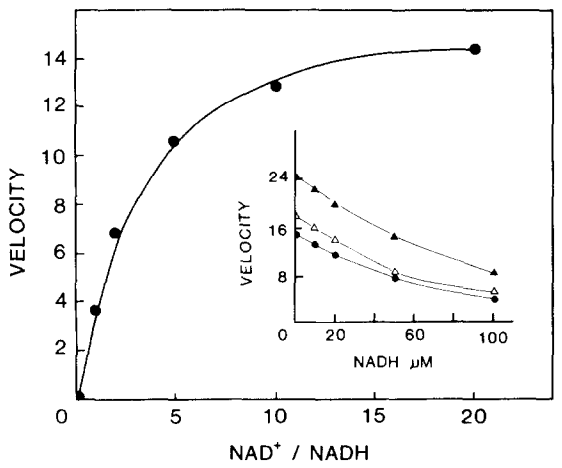

Fig. 1. Response of KGDH to the NAD/NADH ratio. Aliquots of 100 μg enzyme were incubated at pH 7.4 in assay mixtures containing the standard concentrations of KG, TPP, CoA, DTT, EGTA and Tris–acetic acid and the indicated NAD concentrations [0.1 mM (●—), 0.5 mM (—△—), or 1 mM (—▲—) NAD] with increasing concentrations of NADH (0–0.1 mM). The reaction was started by the addition of KG. Velocity units were nmol/min. In the reaction mixture.

Effect of NAD/NADH on the enzyme activity

The effect of the NAD/NADH ratio at pH 7.4 and 0.1 mM NAD with increasing concentrations of NADH (Fig. 1) indicated a decline in the enzyme activity at a NAD/NADH of 5–10. Similar plots with the animal KGDHs from beef heart [19] and bivalve gill tissue [12] showed a decline in activity below a NAD/NADH ratio of 1. The result with the cauliflower KGDH is probably a reflection of the high K_m for NAD (Table 1) and a fairly low K_i for NADH.

The cauliflower KGDH appear to be much more sensitive to anaerobic strees or higher reducing equivalents than the PDH and KGDH activity from animal sources [9, 12]. As the inner mitochondrial NADH oxidases present in the matrix may share a common NADH pool with the NAD-linked enzymes of the Krebs' cycle [1], the inhibition by modest NADH/NAD ratios above one would mean that cauliflower KGDH activity could be reduced considerably during anaerobic stress.

CONCLUSION

With the exception of the inhibition by NADH and the low K_ms for CoA and TTP, the KGDH from cauliflower appears to be fairly similar to the activity in animal mictochondria. The use of the Ultra-Turrax, amphotericin B and the differential centrifugation of the sonicated mitochondria provides a rapid method for the production of an intact preparation of this. important plant mitochondrial enzyme.

EXPERIMENTAL

Cauliflower was purchased from a local store. Chemical reagents were obtained either from Sigma or Fisher.

Mitochondrial preparation. The mitochondria were prepared at 0–4° using a high speed mechanical tissue disruptor. Routinely, 1 kg of the upper cm of cauliflower florets was homogenized in 2 l of a soln containing 0.05 M MOPS (pH 7.4), 1 mM KCl, 5 mM EDTA, 10 mM $MgCl_2$, and 0.6 M sucrose with a Tekmar Ultra-Turrax (Model SD45) for 40 sec at a power setting of 80. The homogenate was filtered through 1 layer of Miracloth (CalBiochem.) and the filtrate pelleted at 8000 g for 15 min. The ppt. was resuspended in 100 ml of 0.05 M MOPS (pH 7) containing 0.1 mM EDTA, 10 mM $MgCl_2$ and 0.4 M sucrose, then centrifuged at 12 000 g for 10 min, and the pellet used as a crude mitochondrial preparation.

Enzyme preparation. Unless otherwise stated all procedures were performed at 2–4°. The mitochondrial pellet from 1 kg of cauliflower florets was resuspended in 100 ml of 0.05 M MOPS (pH 7) containing 0.1 mM EDTA, 1 mM EGTA, 10 mM $MgCl_2$, 0.1 mg/ml trypsin inhibitor, 34 μM leupeptin, 1 mM DTT and 55 μM amphotericin B. This mitochondrial suspension was shaken at 120 rpm for 20 min, at room temp. in a Lab-Line orbital shaker, then centrifuged at 20 200 g for 10 min. The pellet was resuspended in 50 ml of the above buffer in the absence of amphotericin B, and the suspension was sonicated for 10 sec at 3 mA at 0° in 3 ml batches using a Branson Model S75 with the large probe. The sonicated mitochondria were then centrifuged at 20 200 g for 30 min and the supernatant diluted to 90 ml with the above buffer (less amphotericin B) and centrifuged at 150 000 g for 90 min. The pellet was resuspended in 10 ml of a soln containing 20 mM HEPES pH 7.5, 1 mM EGTA, 0.4 M sucrose and 0.1 mM DTT, then incubated overnight at 8°. The soln was then cleared by two centrifugations at 20 000 g for 1 hr; this final supernatant used as the source of enzyme.

Assay procedures. The enzyme activity was assayed spectrophotometrically in a Beckman Model 3600 recording spectrophotometer by measuring NAD reduction at 340 nm at 22°. The standard assay mixture contained the following ingredients: 1.5 mM NAD, 0.3 mM CoA, 0.3 mM TPP, 3 mM DTT, 1 mM EGTA and 0.25 mM KG in 50 mM Tris–HOAc (pH 7.0 or 7.4). A unit of enzyme activity was defined as the amount of enzyme that converted 1 nmol of NAD/min. The kinetic studies were performed as described [20] and the kinetic values were calculated according to a described method [21]. NADH oxidase was assayed in 50 mM MOPS (pH 7.4) with 1 mM NADH. PDH was assyed according to the procedure in ref. [9]. Protein concn was measured by the method of ref. [22] using bovine serum albumin as a standard.

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