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Presence and Regulation of the α-Ketoglutarate Dehydrogenase Multienzyme Complex in the Filamentous Fungus *Aspergillus niger*

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α-Ketoglutarate dehydrogenase has been demonstrated for the first time in cell extracts from the filamentous fungus *Aspergillus niger*. A minimum protein concentration of 5 mg/ml is necessary for detecting enzyme activity, but a maximum of ca. 0.060 μmol/min per mg of protein is observed only when the protein concentration is above 9 mg/ml. α-Ketoglutarate can partly stabilize the enzyme against dilution in the assay system. Neither bovine serum albumin nor a variety of substrates or effectors of the enzyme could stabilize the enzyme against inactivation by dilution. A kinetic analysis of the enzyme revealed Michaelis-Menten kinetics with respect to α-ketoglutarate, coenzyme A, and NAD. Thiamine PP_i was required for maximal activity. NADH, oxaloacetate, succinate, and *cis*-aconitate were found to inhibit the enzyme; AMP was without effect. Monovalent cations including NH_4^+ were inhibitory at high concentrations (>20 mM). The highest enzyme activity was found in rapidly growing mycelia (glucose-NH_4^+ or glucose-peptone medium). We discuss the possibility that citric acid accumulation is caused by oxaloacetate and NADH inhibition of the α-ketoglutarate dehydrogenase of *A. niger*.

The oxidation of α-ketoglutarate within the tricarboxylic acid cycle (TCA cycle) is catalyzed by α-ketoglutarate dehydrogenase (AKGDH) (α-ketoglutarate: lipoate oxidoreductase [acceptor acetylating]; EC 1.2.4.2), which has been isolated as a multienzyme complex from various tissues and organisms (1, 8–10, 13, 14, 24, 25, 34). Previous attempts to demonstrate its presence in filamentous fungi have, however, failed (11, 15, 20, 22), although intact mitochondria from *Aspergillus niger* or *Neurospora crassa* were able to oxidize exogenously added α-ketoglutarate (33, 37).

In our laboratory, the regulation of the TCA cycle in relation to the accumulation of citric acid in *A. niger* has been investigated (15, 16, 19, 29). By measuring metabolite levels, some evidence was obtained for an interruption in the cycle at the α-ketoglutarate dehydrogenase step. However, since we have hitherto failed to isolate the enzyme from this fungus, we were uncertain whether the interruption is caused by repression or by inhibition of its activity. We report here our success in the extraction of this enzyme complex from a citric acid-producing strain of *A. niger* as well as conditions for obtaining reproducible enzyme activities and some of the properties of the enzyme as related to the regulation of the TCA cycle.

MATERIALS AND METHODS

Organism and conditions for growth. The citric acid-producing strain *A. niger* B60 (30) was used throughout these studies. Unless otherwise stated, the organism was kept on potato-dextrose agar slants and cultivated in 1% (wt/vol) glucose medium as described previously (19).

Preparation of cell extracts. Mycelia were harvested by filtration and washed thoroughly with both tap water and distilled water. Because of the high instability of AKGDH, only freshly harvested mycelia were used for enzyme extractions. The mycelial mat was blotted between filter paper sheets to remove excess water and was then ground to a fine powder in a mortar under liquid nitrogen. The powder was then transferred to a 20-ml ultrasonic disintegrator vessel with a triple loop at the bottom to enable circulation of the homogenate; a 2.5-fold volume (vol/wt; mycelial wet weight) of extraction buffer (0.05 M imidazole HCl [pH 8.0], containing 1 mM EDTA, 5 mM 2-mercaptoethanol, 1% [wt/vol] polyvinylpyrrolidone, 30% [wt/vol] glycerol) was added, and the thick suspension was cooled to 2°C in an ice-salt bath. Homogenization was carried out by sonification in a Branson sonifier (adjusted to position 7) for 30 to 40 s. After this, the suspension was carefully stirred by hand until the temperature reached 2°C again. This procedure was repeated 12 to 15 times. The homogenate was then centrifuged at 14,000 × *g* for 25 min at 4°C, and the turbid supernatant was kept for the assay of enzyme activity.

Assay of AKGDH activity. AKGDH activity was assayed by measuring the initial rate of increase in absorbancy at 340 nm in a Pye-Unicam SP 6-450 UV/Vis spectrophotometer, in which the full scale of the recording chart was 1. The standard reaction mixture as optimized during this study contained 100 mM Tris-hydrochloride buffer (pH 7.5), 5 mM 2-mercaptoethanol, 10 mM $MgCl_2$, 2 mM thiamine PP_i, 1 mM sodium α-ketoglutarate, 1 mM NAD, 0.2 mM coenzyme A, (CoA), and enzyme sufficient to produce an increase in absorbancy of 0.02 to 0.2 ΔE/min. α-Ketoglutarate was always pipetted into the cuvette before the addition of the enzyme source. The reaction was then started by the addition of CoA.

Protein determination. Protein was determined by the Comassie blue method (3).

Preparative techniques. Blue dextran Sepharose 4B was prepared as described previously (32); affinity chromatography on blue dextran Sepharose or 5′-AMP Sepharose was carried out in glass columns (16-mm diameter) with a bed height of 40 mm. The columns were immersed into an ice bath during the operation. Not more than 10 mg of protein was applied at one time. Unbound protein was eluted with 0.05 M imidazole buffer (pH 7.5) containing 1 mM EDTA, 5

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TABLE 1. Comparison of various methods for extraction of AKGDH from *A. niger*

Method[a]	Maximum ratio[b]	Maximum protein content	Sp act (μmol/min)
Ball mill[c]	1:8	3.5	—[d]
Ultra Turrax[e]	1:8	2.2	—[d]
Potter-Elvehjem	1:5	5.6	0.027
Ultrasonic disintegration	2:5	14–22	0.068

[a] Each method was carried out under conditions such that the homogenate did not reach temperatures above 4°C. After centrifugation of the homogenates at 14,000 × *g* at 4°C for 25 min, the supernatant was assayed for AKGDH activity.

[b] Maximum ratio of mycelium (wet weight; grams) to milliliters of homogenization buffer which could be handled under adequate temperature control.

[c] Method used 20-g glass beads (0.1 to 0.2 mm [diameter]) per 40 ml of mycelial suspension.

[d] Detection limit of 0.005 U/mg of protein.

[e] Method consisted of 10 10-s treatments with 1-min intervals.

mM 2-mercaptoethanol, and 30% (wt/vol) glycerol at a flow rate of 2 ml/10 min.

Protamine sulfate precipitation was carried out as previously described (28). Polyethylene glycol precipitation was carried out by adding a 25% (wt/vol) aqueous solution dropwise, with stirring to the cell extract, to give a final concentration of 6% (wt/vol).

RESULTS

Extraction of the AKGDH complex from *A. niger*. A variety of methods, which are commonly used for the extraction of mitochondrial enzymes from filamentous fungi like *A. niger*, were tested for their suitability to extract AKGDH activity (see Table 1). Ultrasonic disintegration was finally chosen, since with this method it was possible to homogenize more viscous mycelial suspensions (1 g [wet weight]/2.5 ml of buffer) under adequate temperature control than by other homogenization methods accessible to our laboratory. The protein concentration in the cell-free supernatant was the most important determinant for successful extraction of AKGDH activity (Fig. 1). Thus, a minimal protein concentration above 9 mg/ml was necessary for reproducible extraction. Regardless of the method, no activity could ever be observed in cell extracts containing less than 4 mg of protein per ml. The addition of glycerol (30% [wt/vol]) was not necessary for extraction; however, its addition prolonged the half-life of the activity significantly (from 4 to 55 h) at 4°C. The addition of polyvinylpyrrolidone (1% [wt/vol]) increased the specific activity in the cell extract.

Several combinations of substrates and effectors were also tested for increasing the specific activity of AKGDH in the cell extract when added to the homogenization buffer (e.g., α-ketoglutarate [1 mM], AMP [1 mM], thiamine PP_i [1 mM], $MgCl_2$ [10 mM]), but all of them failed to show an effect.

Assay of AKGDH activity and effect of protein concentration. Since crude extracts of *A. niger* may reduce NAD also in the absence of α-ketoglutarate (although at a low rate), we initially added α-ketoglutarate as the last of the constituents of the assay system. However, only low activities of AKGDH were observed in most cases until we discovered that α-ketoglutarate must be present in the assay before the enzyme is added. The reason for this phenomenon was found to be an exceptional lability of the AKGDH during dilution of the protein moiety; this could be overcome in part by the presence of α-ketoglutarate. When the enzyme was pipetted into the reaction cuvette before the addition of α-ketoglutarate, 70 to 90% of its activity disappeared within 30 s. Thus, in our standard assay (see above), the reaction was always started by the addition of CoA. Among all of the components of the assay system, only α-ketoglutarate was able to stabilize the enzyme, although stabilization was not complete. Even in the presence of 1 mM α-ketoglutarate the enzyme activity remained strongly dependent on the protein concentration in the cuvette (Fig. 2); thus, for a reproducible

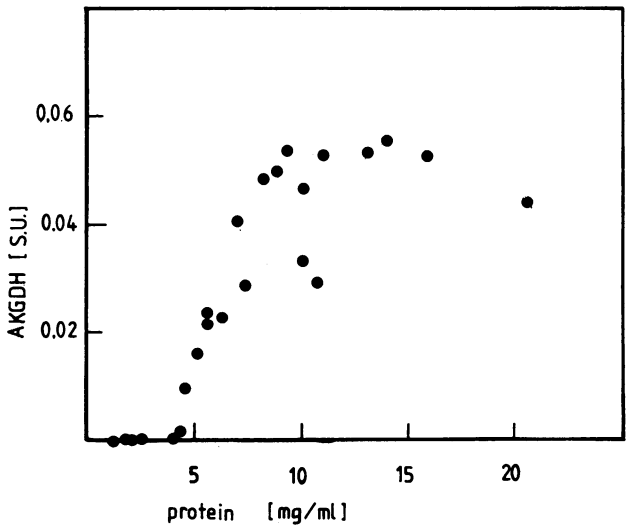

FIG. 1. Effect of protein concentration in the cell extract on the detectability of AKGDH. Each point refers to a single experiment. Various protein concentrations in the extract were reached either by varying the ratio of mycelium (wet weight) to extraction buffer or by varying the time of sonication.

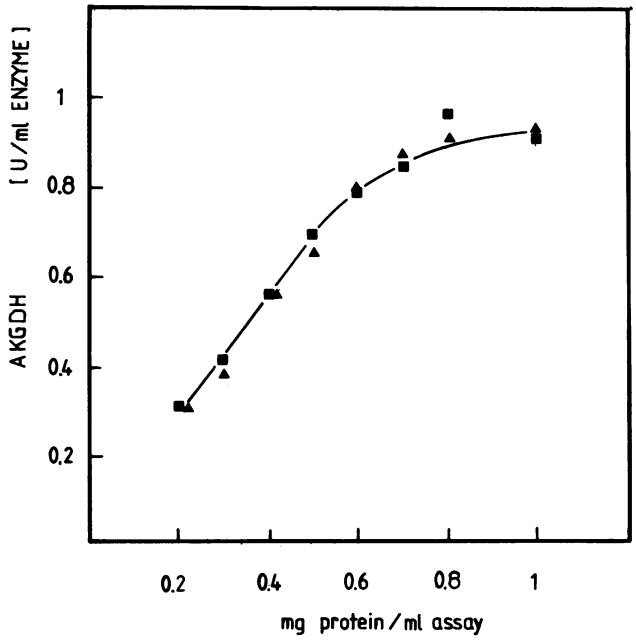

FIG. 2. Influence of the protein concentration in the assay on the activity of AKGDH without bovine serum albumin (■) and in the presence of 0.2% (wt/vol) bovine serum albumin (▲).

assay, care has to be taken to keep the protein concentration constant (usually above 0.4 mg/ml). This effect was also observed in the presence of bovine serum albumin (0.1 to 0.2% [wt/vol]); apparently the concentration of the AKGDH complex in the assay is the critical factor. The following substances did not stabilize the enzyme against dilution: thiamine PP_i (1 mM), $MgCl_2$ (10 mM), AMP (1 mM), and NAD (1 mM). Since we were dealing with a multienzyme complex, we suspected that only one of the components might dissociate from the complex during dilution and become denaturated. Poulsen and Wedding (25) reported dissociation of the lipoyl dehydrogenase from the complex isolated from cauliflower during purification. We thus tried to reactivate AKGDH activity after ca. 50% inactivation by dilution by adding commercially available lipoyl dehydrogenase, but there was no effect.

The pH optimum of the reaction in imidazole buffer was assessed to be above 8.0, but more than 70% of activity was observed within a range of 7.0 to 8.5. Imidazole was found to be the most suitable buffer; Tris gave only 66% of activity.

NADH oxidase, which has been claimed to present a problem in the measurement of AKGDH activity in bacteria (1, 34), was only below 10% of AKGDH activity (usually 0.005 U/mg of protein); thus, enzyme activity could be routinely assayed with NAD as the coenzyme.

Free sulfhydryl groups had to be present in the assay, since otherwise only 21% of activity were observed. 2-Mercaptoethanol and dithioerythritol were equally effective, but glutathione (5 mM) was only 60% as effective.

Attempts to purify the enzyme. Several methods for purifying AKGDH from various sources are available from the literature (9, 10, 13, 24, 25, 28, 36). Preliminary experiments from our laboratory, however, failed to repeat any of them, owing to the high loss of activity when the enzyme becomes diluted to a protein concentration below 5 mg/ml. This problem was only slightly alleviated by the inclusion of 1 mM α-ketoglutarate in the working buffer. On the other hand, when using precipitation or ultracentrifugation techniques, the enzyme can be recovered in the pellet, but only a negligible part of activity can again be solubilized, even with the aid of 0.1% (wt/vol) Triton X-100.

We have also tried to purify the enzyme by blue dextran affinity chromatography or Sepharose 4B gel filtration, at least to some extent, but the losses in activity encountered by these techniques were so great that the final preparations—although their specific activity was increased 3.7-fold in the case of affinity chromatography—had a too low activity per milliliter of sample to be of use for kinetic investigations. Concentration of the sample by means of an Amicon cell was not possible due to the high concentration of glycerol (30% [wt/vol]) needed to stabilize the enzyme. On the other hand, lyophilization of the samples led to a complete loss of activity.

In view of the fact that AKGDH did not bind to blue dextran Sepharose, it is important to note that other proteins like phosphofructokinase or malate dehydrogenase bind to the column under our conditions.

Substrate kinetics. Although we failed to purify the enzyme, we attempted to study some of its kinetic and regulatory characteristics in crude extracts. Figure 3 shows double-reciprocal plots of the reaction rate against α-ketoglutarate, NAD, and CoA at several fixed concentrations of the second substrate and at a saturating level of the third substrate. It is evident from the figure that all plots produce essentially parallel lines. As described previously (31), this is indicative of a "HexaUniPing-Pong" reaction mechanism which has already been recognized as the reaction mechanism of the AKGDH from most sources (27, 34). Separate experiments were carried out to ensure that the range of α-ketoglutarate concentrations was sufficient in any experiment to counteract the loss of activity by dilution. A decrease in the concentration of α-ketoglutarate to 0.05 mM did not alter the curve given in Fig. 2. On the other hand, when the kinetic measurements were carried out with lower concentrations of protein (0.15 mg/ml per assay), only V_{max}, but neither K_m for α-ketoglutarate nor sigmoidity ($n_H = 1.0$), were influenced.

Table 2 lists the K_m values of AKGDH for its substrates as obtained from secondary plots of the results depicted in Fig. 3. The values for thiamine PP_i and Mg^{2+} are also given. Interestingly, cooperative interaction of AKGDH was observed with Mg^{2+}, the Hill coefficient being dependent on the concentration of thiamine PP_i.

Influence of inorganic cations. Monovalent cations (Na^+, NH_4^+, K^+) were inhibitory, but only at concentration exceeding those observed intracellularly (inhibition observed at 50 mM was 48, 70, and 56%, respectively). Mn^{2+} was able to replace Mg^{2+}, but only with 56% of the original activity at 10 mM.

Influence of nucleotide coenzymes. AKGDH from various sources has been reported to be inhibited by NADH (7, 14, 24, 34).

The enzyme from *A. niger* also showed this inhibition. The mechanism was found to be competitive with NAD yielding a K_i of 0.10 mM. Neither NADP nor NADPH exhibited any effect on the activity of the enzyme.

AMP is a potent activator of AKGDH from both microbial (14, 24) and eucaryotic sources (25). However, with the enzyme from *A. niger*, no effect at all could be observed, not even at low concentrations of NAD, CoA, or α-ketoglutarate. ADP also showed no influence. A possible effect of ATP could not be determined, since the enzyme preparation reduced NAD in the presence of ATP at a high rate, and this activity could not be removed by any of the purification procedures tried.

Influence of TCA cycle intermediates. Succinate, *cis*-aconitate, and oxalacetate were shown to be inhibitors of AKGDH. Since oxalacetate has previously been shown to exhibit elevated concentrations during acid fermentation, its mode of action was studied in more detail. Dixon plots yielded a K_i of 0.22 mM, but since no linearity was observed, the value seems to be uncertain. A possible cooperativity of AKGDH was checked by a Hill plot. Slight sigmoidity was observed ($n_H = 2.1$ and 2.3), yielding corresponding $I_{0.5}$ values of 0.24 and 0.38 mM, respectively. In the case of oxalacetate inhibition, it is important to note that the observed inhibition was not due to malate dehydrogenase reoxidizing the formed NADH, since the addition of 0.05 mM NADH in the test system together with oxalacetate did not produce a decrease in absorbance (probably due to NAD inhibition of malate dehydrogenase [19]).

The following intermediates did not inhibit AKGDH activity when tested at the indicated concentrations: citrate (5 mM), isocitrate (0.5 mM), malate (5 mM), fumarate (2 mM), glutamate (5 mM), aspartate (5 mM), and glyoxalate (1 mM).

Formation of AKGDH during growth and influence of carbon and nitrogen sources. Figure 4 shows the specific activities (units per milligram of protein and units per gram of dry mycelium) of AKGDH during growth on 1% (wt/vol) glucose with 0.5% (wt/vol) $(NH_4)_2SO_4$ as the nitrogen source. The activity is highest during rapid growth but rapidly drops during prolonged cultivation.

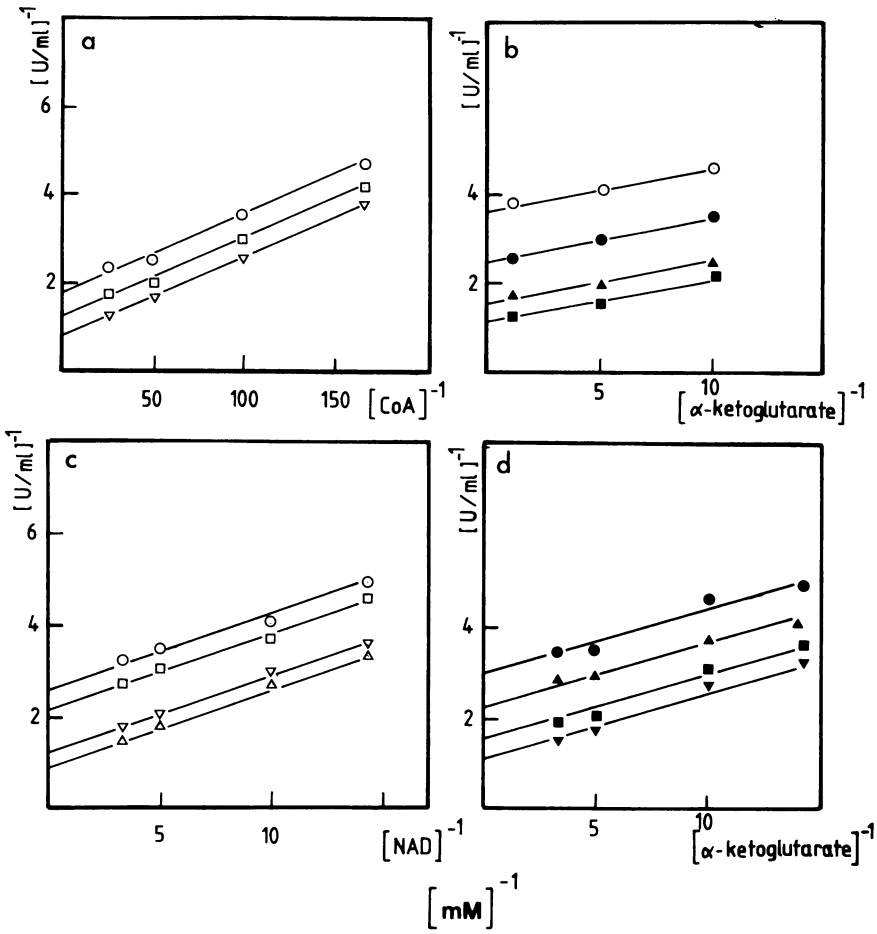

FIG. 3. Lineweaver-Burk plots of AKGDH reaction rates. (a) Affinity for CoA at 1 mM (▽), 0.2 mM (□), and 0.1 mM (○) α-ketoglutarate at a fixed concentration (1 mM) of NAD; (b) affinity for α-ketoglutarate at 40 μM (■), 20 μM (▲), 10 μM (●), and 6 μM (○) CoA at a fixed concentration (1 mM) of NAD; (c) affinity for NAD at 0.3 mM (△), 0.2 mM (▽), 0.1 mM (□), and 0.07 mM (○) α-ketoglutarate at a fixed concentration (0.2 mM) of CoA; (d) affinity for α-ketoglutarate at 0.3 mM (▼), 0.2 mM (■), 0.1 mM (▲), and 0.07 mM (●) NAD at a fixed concentration (0.2 mM) of CoA.

Table 3 shows the specific activities of AKGDH during growth with various carbon and nitrogen sources. Initial experiments showed enhanced activities upon growth on citrate or acetate, especially in the absence of NH_4^+ (29). However, these results were obtained at a time when the influence of the protein and α-ketoglutarate concentrations in the assay had not been recognized. When the protein concentration in the assay was kept constant, however, it was shown that virtually the same activity of AKGDH was obtained under all conditions of growth, given that the mycelia were harvested during the middle of the growth phase. Thus, no induction by TCA cycle acids or influence of nitrogen source is evident.

When the protein concentration in the assay was carefully controlled, the highest activity was obtained upon growth on glucose-ammonium or glucose-peptone medium, whereas the lowest activities were observed on medium containing organic acid, NO_3^-, or a combination of both. However, these findings might not be indicative of repression or induction of AKGDH activity by one of these compounds, but rather may reflect the dependence of AKGDH formation on the growth rate. Low activities are always found in media that favor slow growth (Table 3). Such an assumption would also be compatible with the activity profile from Fig. 4.

We were also interested in assessing the activity of AKGDH during citric acid production by *A. niger*; however, the characteristic pellets which develop during citric acid fermentation completely hindered our attempts to prepare a cell extract with a protein concentration above 4 mg/ml. As a consequence, we were still unable to detect the activity of this enzyme during citric acid accumulation. However, to extrapolate its activity by examining the influence of medium components on its activity, the influence of at least one factor, sugar concentration (which is high during citric acid

TABLE 2. Kinetic properties of AKGDH from *A. niger*.

Sample	K_m values (mM) determined by HexaUniPing-Pong[a] reaction mechanism
α-Ketoglutarate	0.4
CoA	0.02
NAD	0.25
Thiamine PP_i	0.3 (uncertain; thiamine PP_i partially bound to AKGDH)
Mg^{2+}	Not determinable, since sigmoidity is apparent ($S_{0.5}$ = 2.3 mM; n_H = 1.7)

[a] Calculated from the data shown in Fig. 3 as described by Rudolph and Fromm (31).

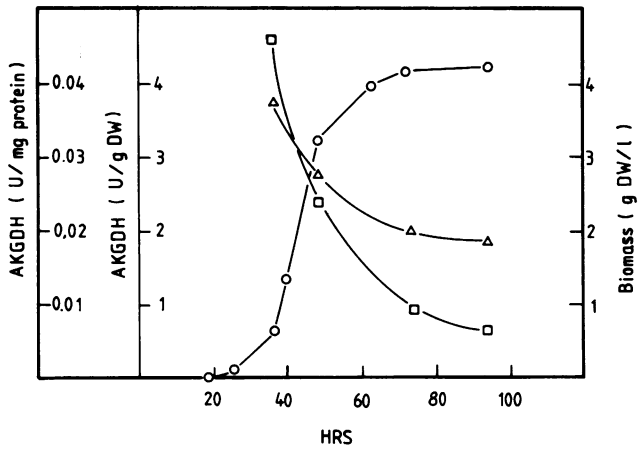

FIG. 4. Formation of AKGDH during growth of *A. niger* on glucose-NH_4^+ medium. Symbols: (○) growth (dry weight); (△) AKGDH specific activity; and (□) AKGDH activity per gram (dry weight) of mycelium.

fermentation [29]), on AKGDH activity was tested. Apparently, glucose in the range of 1 to 3% (wt/vol) does not influence AKGDH activity; however, in the presence of 10% (wt/vol) glucose which is usually used in citric acid fermentation, the specific activity dropped to only 23% (0.013 U/mg of protein). This was not caused by a lower protein content during extraction. Also, in this case, formation of AKGDH may be influenced by the growth rate.

DISCUSSION

The present paper is the first to describe the presence and some of the kinetic and regulatory properties of the AKGDH complex of *A. niger*. Previous failures to detect this enzyme in *A. niger* and other fungi (11, 15, 20, 22) may have been caused by its striking lability during dilution or its requirements for α-ketoglutarate for stabilization in the assay system. These properties have not been reported before and it seems probable that many workers may have overlooked them. Although a scrutinization of the assay methods used revealed that most workers started the reaction by the addition of CoA or NAD, it has never been indicated whether α-ketoglutarate was added before the addition of the enzyme source. The protection of the enzyme by α-ketoglutarate against dilution in the assay system might also help to explain the observation of low levels of activity in citrate- or acetate-grown mycelia (20) and our previous findings that AKGDH can be found after NH_4^+ exhaustion from the medium (29). Under such conditions, the cellular α-ketoglutarate pool rises appreciably (0.5 to 1 μmol/g of dry mycelium); thus, a certain amount of α-ketoglutarate is transferred into the cell extract during homogenization, which could amount to 0.04 mM (by assuming total extraction at a wet weight/buffer ratio of 1:5). Thus, within the assay system, 0.01 mM concentrations of α-ketoglutarate could be present, which might be responsible for a partial stabilization.

We have also tested some other fungi, particularly those which have been reported to be devoid of the enzyme (i.e., *Aspergillus nidulans* and *Penicillium chrysogenum* [20]); but in each case, activity could be extracted, provided that the precautions described in this paper were considered. While this work was at its final stage, Osmani and Scrutton (23) detected AKGDH by solubilization of mitochondria from *A. nidulans* with Nonidet P-40. We concluded, therefore, that the enzyme is probably present in fungi, and all contrary reports may have been caused by its unusual lability.

The lability of AKGDH against dilution seriously interfered with all attempts to purify the enzyme, although several approved procedures for purification of the enzyme complex from eucaryotic tissues have been applied. A similar lability has been found for a multienzyme aggregate carrying out valine biosynthesis from pyruvate in *N. crassa* homogenates (12), but these authors reported no attempts to purify the aggregate. Theoretically, procedures involving precipitation (by ultracentrifugation or polymer addition; 8–10, 13, 24, 25, 36) should not be affected by the lability of the enzyme against dilution. In fact, AKGDH activity could be precipitated by ultracentrifugation with an acceptable yield. However, in this case, a resolubilization was not possible, even by the use of detergents. The reason for this behavior is unclear at present, but we speculate that it is related to artificial formation of vesicles during ultrasonic disruption of the mycelia which aggregate and thereby adsorb or trap AKGDH during precipitation. A clarification of this aspect would be needed before future attempts to purify the enzyme.

Concerning the regulation of the enzyme from *A. niger*, it is evident that the results obtained with crude cell extracts should be interpreted with caution. This holds especially for the findings of mere Michaelis-Menten kinetics with α-ketoglutarate, CoA, and NAD and the lack of activation by AMP. Recently, the lack of activation of some enzymes from filamentous fungi by commonly observed activators has been reported by some laboratories (e.g., pyruvate kinase and FDP [2], phosphoglycerate mutase and 2,3-phosphoglycerate [26], and malate dehydrogenase and FDP [19]). However, in at least one case, this could be attributed to a tight binding of the activator to the enzyme during extraction (e.g., pyruvate kinase and FDP [21]). If tight binding of AMP to AKGDH from *A. niger* is assumed, then a lack of activation by AMP addition and also the observation of Michaelis-Menten kinetics becomes plausible. In *Acetobacter xylinum*, AMP changes the kinetics of AKGDH with α-ketoglutarate from sigmoidal to hyperbolical (14). However, in other cases, Michaelis-Menten kinetics with α-ketoglutarate has been observed despite the presence of AMP (24, 25, 27). In this context, it should be especially noted that a possible loss of AMP during purification procedures involving the dilution of the protein cannot be the cause of the apparent lability of the enzyme, since the addition of AMP to the buffers had no noticeable effect on the stability of AKGDH.

TABLE 3. Formation of AKGDH during growth of *A. niger* on media with various carbon and nitrogen sources[a]

Medium	Sp act[b]	Approx growth rate at the point of harvest[c]
Glucose-NH_4^+ (10:5 [g/g])	0.062	0.15
Glucose-NO_3^- (10:8 [g/g])	0.012	0.028
Citrate-NH_4^+ (10:5 [g/g])	0.011	0.020
Citrate-NO_3^- (10:8 [g/g])	0.010	0.018
Glucose-peptone (10:5 [g/g])	0.066	0.20
Glucose-glutamate (10:5 [g/g])	0.034	0.10

[a] Values calculated from at least three separate determinations.

[b] Assayed under conditions in which the protein content of the assay system was 0.4 mg/ml. Values are given in micromoles per minute per milligram of protein.

[c] Determined by assuming a linear increase in dry weight between the point of complete germination and the point at which ca. 50% of total biomass has formed (point of harvest). Values are given in grams per liter per hour.

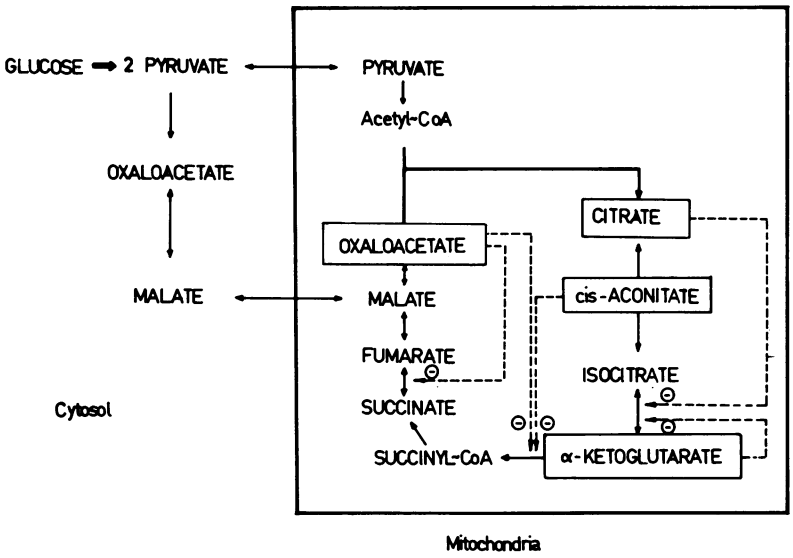

FIG. 5. Regulatory properties of the TCA cycle in *A. niger* as related to the accumulation of citric acid. Data used are from the present paper and as described previously (29).

Among a variety of metabolites related to the TCA cycle, oxalacetate, *cis*-aconitate, succinate, and NADH were shown to be inhibitors of the enzyme from *A. niger*. A critical interpretation of this finding clearly depends on a knowledge of the in vivo concentrations of these metabolites in *A. niger*, especially in the mitochondrial compartment. Although we have previously measured the total mycelial levels of most of the metabolites related to energy metabolism in *A. niger* during citric acid fermentation (4–6, 15), we are still unaware of their mitochondrial concentrations, since appropriate methods for separation of organelles under preservation of in vivo metabolite concentrations as are optimized for mammalian tissues are still not applicable to filamentous fungi.

Nevertheless, oxaloacetate and NADH are the most likely candidates for exerting an inhibitory influence on AKGDH activity and thereby triggering citric acid accumulation, since both intermediates have previously been shown to display elevated intracellular concentrations during the initial phase of citric acid fermentation (4, 15, 29), whereas succinate and *cis*-aconitate do not. It is interesting that the same metabolites have been implicated in the control of the TCA cycle in higher eucaryotes (18, 35, 38), although oxaloacetate control of AKGDH has not been reported from these tissues.

In summary, we concluded that the metabolic block at the AKGDH step is probably caused by the inhibition of the enzyme and not by its repression. We are therefore able to give a more detailed interpretation of the metabolic events leading to citric acid accumulation. In view of other relevant regulatory aspects of the cycle (cf. ref. 29), its regulation might be interpreted as presented in Fig. 5. Once pyruvate carboxylase activity increases and the oxalacetate pool rises, the TCA cycle becomes interrupted at the AKGDH and succinate dehydrogenase step. As a consequence, the cycle does not operate cyclically, and several intermediates accumulate according to the equilibria of the enzymes involved. Since citrate inhibits its own catabolism (29) but is not able to decrease the supply of acetyl-CoA or oxalacetate by glycolysis (5, 6, 21), and since the equilibria and affinities of succinate dehydrogenase, fumarase, and malate dehydrogenase favor the formation of citrate (15, 19), it is citrate which can finally be accumulated to an appreciable amount.

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