THE *iv-3* MUTANTS OF *NEUROSPORA CRASSA*
II. ACTIVITY OF ACETOHYDROXY ACID SYNTHETASE[1]

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Received November 22, 1968

IN the preceding paper (KUWANA and WAGNER 1969) it is shown that the *iv-3* mutants accumulate pyruvate when growing in the presence of isoleucine and valine. Previously we had also shown that mitochondrial fractions and supernatants prepared from these mutants are unable to convert pyruvate to valine *in vitro*, but do convert α-acetolactate to valine (WAGNER, BERGQUIST, BARBEE and KIRITANI, 1964). The mitochondrial fraction of wild type, however, readily converts pyruvate to valine (KIRITANI, NARISE, BERGQUIST and WAGNER, 1965). These facts make it highly probable that the *iv-3* mutants are blocked at the step at which pyruvate and α-ketobutyrate are condensed with "active" acetaldehyde to form α-acetolactate and α-aceto-hydroxybutyrate, the two acetohydroxy acid precursors of valine and isoleucine, respectively. Hence, it should be expected that the enzyme catalyzing this step, acetohydroxy acid synthetase, should be altered in activity or missing in the *iv-3* mutants. In an earlier paper (WAGNER, BERGQUIST, BARBEE and KIRITANI 1964) this enzyme was reported to be present in the *iv-3* mutants at about the same level as in wild type.

In this communication it is shown that most of the activity previously measured in the *iv-3* mutants and part of the activity measured in wild type is due to an acetoin-forming system in the whole cell extract. It is also shown that fresh extracts of wild-type mitochondria contain a labile acetohydroxy acid synthetase with a pH optimum of about 7.6 which is entirely missing in the *iv-3* mutants. In addition, both fresh and aged extracts of the mutant mitochondria and aged extracts of wild-type mitochondria contain an acetohydroxy acid synthetase activity with a relatively low specific activity. The enzyme present in aged wild-type extracts has been purified and shown to have a pH optimum of 6.0–6.5 (KUWANA, CAROLINE, HARDING and WAGNER 1968).

MATERIALS AND METHODS

The wild-type KJTA and *iv-3* mutants of *Neurospora crassa* employed in these experiments are described by KUWANA and WAGNER (1969). The *iv-2* mutant 320a was obtained as described by WAGNER, SOMERS and BERGQUIST (1960).

Conidia were inoculated in VOGEL's medium (VOGEL 1956) supplemented with 4μmoles/ml

[1] Work supported by grants from the NIH (GM-12323) and the Robert Welch Foundation of Houston, Texas.
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each of L-valine and L-isoleucine where indicated. The mycelium was grown in flasks on a rotating shaker at 29°C for 18 hrs. The leaky *iv-3* mutant, 355A, was grown for 64 hrs when no supplement was added. The mycelium was harvested on a Buchner funnel, washed with 0.1M Tris-HCl at pH 7.8 in 0.1M sucrose then ground with 1.5 times its weight of acid-washed sand and an equal volume of a solution containing 0.25M sucrose and 0.15% bovine serum albumin. The ground homogenate was centrifuged twice at 1,500 × *g* for 15 min to remove the sand and cell debris. The resulting supernatant was then centrifuged at 12,000 × *g* for 30 min and the second supernatant carefully removed with a syringe. The pellet, which contains essentially all of the mitochondria, was washed with 0.25M sucrose plus 0.15% bovine serum albumin solution and then either extracted for 10 min with 0.3% sodium deoxycholate (DOC) in 0.02M Tris-HCl at pH 8.5, or suspended in 0.1M Tris pH 8.0, 0.1M sucrose and sonicated in a Branson sonifier for 20 sec at 4 amps. These suspensions were centrifuged at 39,000 × *g* for 10 min and the resulting supernatants were used in these experiments. These pellet extracts contain the acetohydroxy acid synthetase activity originally present in the pellet centrifuged down at 12,000 × *g*. When whole mitochondrial pellets were assayed, the 12,000 × *g* pellets were resuspended in 0.25M sucrose and 0.15% BSA. The supernatant assayed for acetoin-forming activity was obtained by centrifuging the 12,000 × *g* supernatant at 39,000 × *g* for 30 min to remove any remaining mitochondria.

The acetohydroxy acid synthetase activity was assayed with pyruvate as substrate using the procedure described by KUWANA, CAROLINE, HARDING and WAGNER (1968) and the Westerfeld test for acetoin (WESTERFELD 1945). In this method free acetoin is measured in NaOH-stopped reactions and α-acetolactate is measured from the difference in optical densities between acid and base-stopped reactions. Potassium phosphate buffers at a concentration of 0.1M in the assay were used over a pH range of 5.7 to 8.0. The reactions were stopped with 0.1ml of 2M $ZnSO_4$ and 0.1ml 2M NaOH as described by SATYANARAYANA and RADHAKRISHNAN 1963). Specific activities were calculated as μmoles acetolactate or acetoin per mg protein per hr. Protein was determined by the Folin-Ciocalteau test (LOWRY *et al.* 1951).

The purification of the acetohydroxy acid synthetase activity from stored DOC pellet extract was carried out by the procedure described by KUWANA, CAROLINE, HARDING and WAGNER (1968).

RESULTS

Activity of the acetohydroxy acid synthetase in the wild type: Wild-type DOC pellet extracts prepared as soon as possible after harvesting the mycelium have an acetohydroxy acid synthetase with a pH optimum of about 7.6. However, upon heating the extract at 37°C for 30 min, or storing it at 4°C overnight, a considerable amount of activity is lost, and the pH optimum in the basic range disappears (Figure 1). In some experiments activity was found only below pH 7.0 in aged extracts. This loss of activity is prevented by adding the strong reducing agent dithiothreitol to DOC extracts. Details of the conditions for stabilization of the activity will be described elsewhere.

In Table 1 it is shown that the basic pH acetohydroxy acid synthetase activity is not affected by the extraction procedure used. Mitochondrial extracts made by DOC treatment or by sonication show about the same activity as resuspended whole mitochondria. Similar results were found with the *iv-3* mutant 344A. There does seem to be some loss of activity in the mutant upon extraction; however, the activity in the whole pellet is still considerably lower than wild type. The basic pH acetohydroxy acid synthetase in wild type is not found to be repressed by isoleucine and valine as can be seen from Table 2.

Activity of the synthetase in the mutants: The DOC pellet extracts of all *iv-3* mutants examined were found to be deficient in basic pH acetohydroxy acid

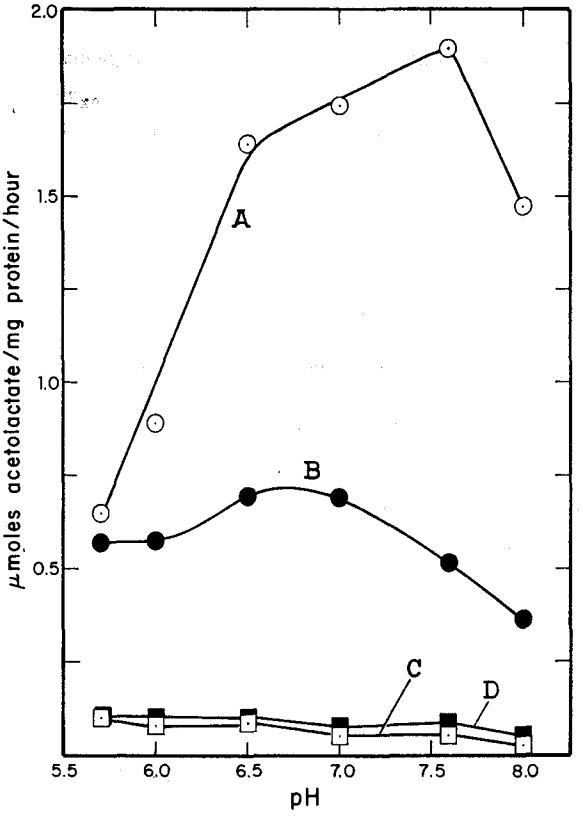

FIGURE 1.—pH curves of the acetohydroxy acid synthetase activity in DOC extracts of the 12,000 × *g* pellets obtained from wild type and the *iv-3* mutant, 344A. Both wild type and 344A were grown on minimal supplemented with L-isoleucine and L-valine. (A) fresh DOC extract of wild-type pellet. (B) DOC extract of wild-type pellet after incubation at 37°C for 30 min. (C) fresh DOC extract of 344A pellet. (D) DOC extract of 344A pellet after incubation at 37°C for 30 min.

TABLE 1

Effect of extraction on acetohydroxy acid synthetase activity

Strain	Locus	Supplement*	Isoleucine-valine requirement	Type of extract	μmoles α-acetolactate/mg protein/hr	
					pH 6.0	pH 7.6
KJTA	wild type	none	—	whole mitochondria	0.72	0.97
KJTA	wild type	none	—	sonicate	0.98	1.07
KJTA	wild type	none	—	deoxycholate	0.76	1.40
344A	*iv-3*	isoleucine-valine	+	whole mitochondria	0.28	0.21
344A	*iv-3*	isoleucine-valine	+	sonicate	0.09	0.15
344A	*iv-3*	isoleucine-valine	+	deoxycholate	0.07	0.05

* 4mM each of L-isoleucine and L-valine where indicated.

TABLE 2

Activity of acetohydroxy acid synthetase in wild type and iv-3 *mutants*

The activities were determined in freshly prepared deoxycholate extracts of 12,000 × *g* pellets.

Strain	Locus	Supplement*	Isoleucine-valine requirement	μmoles α-acetolactate/mg protein/hr	
				pH 6.0	pH 7.6
KJTA	wild type	none	none	0.76	1.40
KJTA	wild type	isoleucine-valine	none	0.88	1.91
364A	*iv-3*	isoleucine-valine	complete	0.06	0.06
346A	*iv-3*	isoleucine-valine	complete	0.11	0.11
354A	*iv-3*	isoleucine-valine	complete	0.16	0.09
344A	*iv-3*	isoleucine-valine	complete	0.07	0.05
357a	*iv-3*	isoleucine-valine	complete	0.19	0.11
353A	*iv-3*	isoleucine-valine	partial	0.26	0.16
355A	*iv-3*	isoleucine-valine	partial	0.20	0.13
355A	*iv-3*	none	partial	0.19	0.11
320a	*iv-2*	isoleucine-valine	complete	0.67	0.98

* 4mM each of L-isoleucine and L-valine where indicated.

synthetase as shown in Table 2. None showed a complete absence of activity, but all show only a fraction of the wild-type activity at pH 7.6. Two of the mutants tested, 353A and 355A, were leaky strains which do not accumulate large amounts of pyruvate (KUWANA and WAGNER 1969). They have, nonetheless, very low synthetase activities at pH 7.6 just as the mutants which are tightly blocked. One of these leaky mutants, 355A, was also grown on minimal medium with no isoleucine or valine. This was not found to affect the low level of acetohydroxy acid synthetase specific activity observed in this mutant.

It should be noted that the residual synthetase activity in the fresh crude extracts from the mutants shows no pH optimum at the concentrations assayed. In addition, the residual activity of the extract from mutant 344A and others is not affected significantly by heating for 30 min at 37°C (Figure 1) unlike the activity present in fresh DOC extracts obtained from wild type. Lack of pH 7.6 acetohydroxy acid synthetase activity in the mutants cannot be due to the presence of an inhibitor, since mixing DOC pellet extracts from wild type and several *iv-3* mutants clearly showed that the pH 7.6 wild-type activity was unaffected by the presence of mutant extracts.

Another mutant *iv-2* (320a) which is known to lack the second enzyme in the biosynthetic pathway for valine and isoleucine (WAGNER, BERGQUIST, BARBEE and KIRITANI 1964) was found to have normal pH 7.6 acetohydroxy acid synthetase activity unlike any of the *iv-3* mutants as shown in Table 2.

Properties of the partially purified synthetases in aged pellet extracts: DOC pellet extracts that had been kept in the cold overnight were used to purify the mutant and the wild-type enzyme. Table 3 gives the results obtained with five of the *iv-3* mutants and the wild type. It will be noted that the specific activity was essentially the same for the mutants and the wild type in the aged DOC pel-

TABLE 3

Purification results and properties of purified acetohydroxy acid synthetase in aged extracts from five iv-3 *strains and the wild type*. .

Purification step	KJT1960A	Specific activity (μmoles α-acetolactate formed/mg/hr at pH 6.5) 344A	353A	357A	364A	Y7110A
pellet extract	0.12– 0.32	0.25	0.19	0.27	0.17	0.22
40 to 70% ammonium sulfate fraction	0.87– 1.65	1.46	1.31	1.76	1.55	1.39
Sephadex G-100 fraction	2.02– 5.88	4.77	3.02	4.11	4.58	3.06
Sephadex—DEAE A-50	19.0–39.5	41.5	19.9	42.7	37.0	20.8
final purification	107–212×	166×	105×	158×	218×	95×
final yield percent	12–28	17	18	18	17	12

The pellet extract was obtained by treatment with sodium deoxycholate overnight at 4°C. Purification was carried out as described previously (KUWANA, CAROLINE, HARDING and WAGNER 1968).

let extracts and in the purified final fractions. The final yields were also in the same range. Thus the residual acetohydroxy acid synthetase activities in these aged mutant and wild-type extracts, which also have no significant activity at pH 7.6, purify in the same manner.

Activity of the acetoin-forming system: The pellet obtained by centrifugation of the fresh, crude supernatant at 12,000 × *g* contains essentially all of the pH 7.6 acetohydroxy acid synthetase activity. The 39,000 × *g* supernatants of wild type and the *iv-3* mutants synthesize acetoin and a negligible amount of acetolactate in the presence of pyruvate. Acetaldehyde can also be used as a substrate for this reaction. This acetoin-forming activity has a pH optimum in the acid range and is not affected by heating at 37°C for 30 min (Figure 2). Unlike the acetohydroxy acid synthetase, this enzyme is repressed by more than 60% when wild type is grown on isoleucine and valine. The repressed activity is not affected by heat treatment (Figure 2). All of the *iv-3* mutants tested have this activity. There was some variation among the mutants but the activities ranged between those found for wild type when grown on supplemented or unsupplemented medium.

DISCUSSION

Three different enzymic activities, as measured by the Westerfeld test for acetoin, can be distinguished in extracts of wild-type *Neurospora crassa*. Two of these are found in the 12,000 × *g* pellet which is primarily composed of mitochondria (GREENAWALT, HALL and WALLIS 1967), and both of these activities form α-acetolactate from pyruvate. The third activity is found in the 39,000 × *g* supernatant and forms free acetoin from pyruvate.

Fresh, crude deoxycholate extracts of the 12,000 × *g* pellet show activity for the formation of α-acetolactate from pyruvate with a pH optimum of about 7.6. The *iv-3* mutants have very low acetohydroxy acid synthetase activity at pH 7.6; therefore, it is believed to be the enzyme activity involved in the biosynthesis of

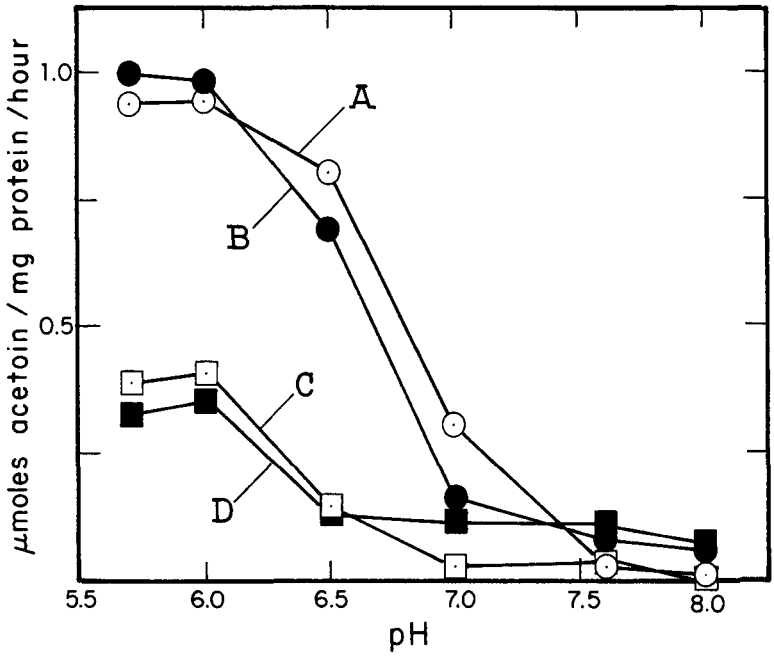

FIGURE 2.—pH curve of the acetoin-forming activity in the 39,000 × *g* supernatant of wild type. (A) 39,000 × *g* supernatant from wild type grown on minimal. (B) Same as A after incubation at 37°C for 30 min. (C) 39,000 × *g* supernatant from wild type grown on minimal supplemented with L-isoleucine and L-valine. (D) Same as C after incubation at 37°C for 30 min.

isoleucine and valine *in vivo*. The location of this synthetase in the pellet is in agreement with this being the active synthetase in valine synthesis, since we had previously shown that the overall synthesis of valine from pyruvate is in the mitochondrial fraction (KIRITANI *et al.* 1965).

The second pellet activity is observed in the wild type after aging of the pellet extracts. Aging results in a significant loss of α-acetolactate-forming activity and the disappearance of the basic pH optimum. The residual activity observed purifies in a similar manner as the activity present in aged extracts of several of the *iv-3* mutants. HALPERN and UMBARGER (1959) have reported the existence of two apparent synthetases in *Aerobacter aerogenes*. One has a pH optimum of 6.0, and the acetolactate formed by this enzyme is decarboxylated to acetoin by acetolactate decarboxylase. The other synthetase has a pH optimum of 7.5–8.0 and is believed to be the enzyme involved in isoleucine-valine biosynthesis as indicated by mutant studies (HALPERN and EVEN-SHOSHAN 1967). In yeast MAGEE and ROBICHON-SZULMAJSTER (1968) found an acetohydroxy acid synthetase with a pH optimum of 7.2 which is present in negligible amounts in an isoleucine-valine requiring mutant.

The Neurospora 39,000 × *g* supernatant enzyme described here has a pH optimum in the acid range and forms free acetoin from pyruvate almost exclusively. MAGEE and ROBICHON-SZULMAJSTER (1968) also described an acetoin-

forming system in yeast with a pH optimum of about 6.5. Their mutant which lacks acetohydroxy acid synthetase has more free acetoin formation than wild type. This does not seem to be the case in Neurospora where the levels of activity for formation of free acetoin in the *iv-3* mutants are in the same range as the wild type. In addition, in Neurospora all acetohydroxy acid synthetase activity was shown to be present in the mitochondria while the acetoin-forming system was found in the 39,000 × *g* supernatant.

It is not yet clear whether the Neurospora supernatant enzyme described here forms acetolactate as an intermediate as is the case with Aerobacter (JUNI 1952). An alternative possibility is that pyruvate is directly decarboxylated to form active acetaldehyde and acetaldehyde which then condense to form acetoin as has been shown with yeast (JUNI and HEYM 1968).

Another important consideration presently under investigation is whether there is any relationship between the acetoin-forming activity in the 39,000 × *g* supernatant and either of the 12,000 × *g* acetohydroxy acid synthetase activities. It is of particular interest to determine the significance of the finding that supernatant enzyme is repressed when wild type is grown on isoleucine-valine while the pH 7.6 pellet synthetase is not.

SUMMARY

Acetohydroxy acid synthetase activity with a pH optimum around 7.6 was found to be present in fresh DOC extracts of wild-type crude mitochondrial pellets. There was shown to be a loss in synthetase activity at pH 7.6 as a result of storage of such an extract overnight in ice or incubation at 37°C for 30 min. In corresponding fresh pellet extracts obtained from the *iv-3* mutants, the acetohydroxy acid synthetase activity is present at a much lower level than that of fresh wild-type extracts. The level of this residual activity in the *iv-3* mutants does not change as a result of aging. The 12,000 × *g* pellet synthetase activities measured in aged wild-type extracts and in aged extracts obtained from five *iv-3* mutants were shown to purify in a similar manner. It is concluded that the block in the *iv-3* mutants is a result of deficient activity of the basic pH synthetase. Acetoin-forming activity was shown to be present in the 39,000 × *g* supernatants of wild type and the *iv-3* mutants. This activity was shown to be repressed in wild type grown on minimal medium supplemented with isoleucine and valine. The acetoin-forming activity was shown to be stable with incubation at 37°C for 30 min whether obtained from wild-type mycelium grown on minimal medium or from mycelium grown on minimal medium supplemented with isoleucine and valine.

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