NEUROSPORA MUTANTS WITH MITOCHONDRIA DEFICIENT IN DIHYDROXY ACID DEHYDRATASE. PROPERTIES OF DIHYDROXY ACID DEHYDRATASE FROM MUTANT STRAIN 332.

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SUMMARY

Dihydroxy acid dehydratase from iv-1 mutant strain 332 of Neurospora crassa has been found to differ from the wild-type enzyme with respect to heat-inactivation, pH-dependence, and kinetics. These findings suggest that the altered structure of this enzyme is the basic defect hindering its incorportation into mitochondria.

INTRODUCTION

In Neurospora crassa the synthesis of valine and isoleucine from pyruvate and α-ketobutyrate requires four enzymes, including dihydroxy acid dehydratase (DHAD, EC 4.2.1.9), and occurs primarily if not entirely in the mitochondria (1). The step catalyzed by DHAD, the conversion of the dihydroxy precusors to the α-keto precursors of valine and isoleucine, is blocked in the valine-isoleucine requiring mutants of Neurospora crassa which map at the iv-1 locus (2) even though homogenates of these mutants contain appreciable amounts of DHAD (2,3). The cause of the metabolic block in the iv-1 mutants has been explained, at least in part, by the recent finding (3) that mitochondria of these mutants contain relatively very little DHAD. Nearly all of the DHAD in homogenates of the iv-1 mutants is found in the soluble fraction obtained by centrifugation at 35,000 x g for 30 min. In contrast, the mitochondrial fraction of homogenates of the wild-type contains 60-80% of the total DHAD.

The results of an earlier study (4) indicate that the DHAD in the soluble and mitochondrial fractions of homogenates of the wild-type strain of Neurospora used in

this study are the same molecular species. This conclusion is further supported by the results of recent genetic and biochemical studies (5). If Neurospora does indeed contain only one molecular species of DHAD and if it is synthesized outside of the mitochondria as we now believe (6) it follows that this enzyme must be transported in some way from its site of synthesis to its site(s) of localization in the mitochondria. It is now generally accepted that 90-95% of mitochondrial protein is synthesized outside of the mitochondria (7,8), but little if anything is known about the actual mechanism(s) by which a protein synthesized outside the mitochondria is incorporated into the organelles. Undoubtedly the process would be influenced by the structure of the protein itself. Therefore it is conceivable that an alteration in the structure of DHAD could cause a block in the incorporation of DHAD into the mitochondria.

The purpose of this study was to determine if the properties of the DHAD from an iv-1 mutant are altered, indicating that the structure of DHAD has been altered by mutation at the iv-1 locus. To accomplish this, preparations of DHAD from an iv-1 mutant and from the parental wild-type strain were compared with respect to heat-inactivation, pH dependence, and kinetics.

MATERIALS AND METHODS

Mutant strain 332 (2) and wild-type, strain KJT 1960A (9) were grown in carboys containing 8 liters of Vogel's minimal medium (10) supplemented with 64 mMoles each of DL-valine and DL-isoleucine. KJT 1960A was also grown in minimal medium without supplement.

Mycelium was grown at 30± 1°C under vigorous forced aeration for 30 hr and then was harvested, washed, and homogenized by procedures described previously (3) with the exception that the mycelium was washed and homogenized in a buffer instead of a sucrose solution. The buffer, pH 9.0, contained 50 mM Tris-HCl and 10 mM $MgSO_4$ (buffer A).

The homogentate was centrifuged at 1,000 x g for 15 min and the resulting supernatant was centrifuged at 35,000 x g for 30 min to yield the crude supernatant. Preparations of DHAD from the mutant and wild-type were obtained by fractionation with ammonium sulfate (Special Enzyme Grade, Mann Research Laboratories). A saturated solution of ammonium sulfate containing 50 mM Trizma base was added dropwise to crude supernatant at 5°C. Precipitated material was collected at 20%, 40%, 60%, and 75% saturation by centrifugation at 35,000 x g for 15 min. The pellets obtained by centrifugation were dissolved in buffer A. The fraction precipitating between 40% and 60% saturation was used as the enzyme preparation from strain 332. The fraction precipitating between 60% and 75% saturation was used as the preparation of wild-type enzyme.

DHAD was assayed by the method described by Altmiller and Wagner (4) with the exception that the incubation buffer (buffer B) contained 100 mM Tris-HCl instead of 50 mM. The substrate α,β -dihydroxyisovalerate (DHV) was obtained from Reef Laboratory, Lafayette, Indiana. Protein concentration was estimated by the method of Lowry et al. (11) using bovine serum albumin as a standard.

In the thermal-denaturation experiments enzyme was mixed with buffer B, pH 8.3, and incubated in an ice-water bath for 5 min immediately prior to the heat-incubation. Enzyme preparations were incubated for 20 min at various temperatures in the range, 37°C to 64°C. Immediately following the heat-incubation the tubes containing buffer and enzyme were cooled for 2 min in an ice-water bath. The heat-incubated preparations and appropriate unheated controls were then assayed for DHAD activity.

RESULTS

The pH optima for activity with the substrate DHV are seen in figure 1 to be about 7.8 and 8.2 for DHAD from strain 332 and the wild-type, respectively. The pH optimum for activity of the wild-type enzyme with DHV was reported previously to be approximately 7.9 (4,12). However, the pH of the buffers used in those experiments was determined with a linen-fiber junction electrode and this type electrode is known to

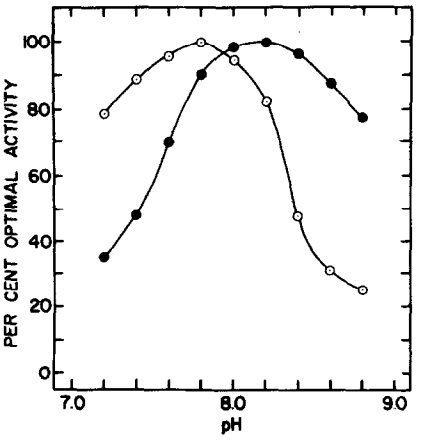

Fig. 1.

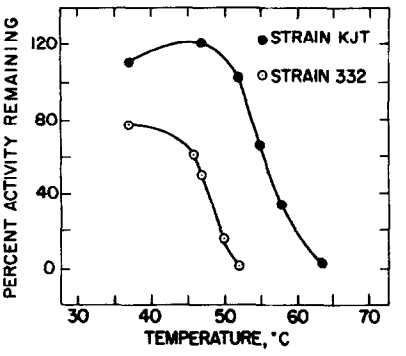

Fig. 2.

Figure 1. Effect of pH on the production of α-keto acids by preparations of DHAD from Neurospora mutant strain 332 (open circles) and wild-type (closed circles). The pH values were obtained with buffer B. Each assay mixture contained 250 ug protein and 10 micromoles DHV in a volume of 1 ml.

Figure 2. Thermolability of DHAD from Neurospora mutant strain 332 (open circles) and wild-type (closed circles). The enzyme preparations were heated for 20 min at the indicated temperatures. Each assay mixture contained 250 ug protein and 10 micromoles DHV in a volume of 1 ml.

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give erroneous pH values with Tris-HCl buffers (13). The pH of the buffers used in the present study was determined with a Model 26 Radiometer pH meter equipped with a Radiometer electrode, type GK 2321C. This electrode gives accurate pH values with Tris-HCl buffers (14). Trizma standards (Sigma Chemical Company) were used to calibrate the pH meter.

Figure 2 reveals that the DHAD from mutant strain 332 is more thermolabile than the enzyme from the wild-type. The temperature inactivation coefficient (T_i), which is defined as the temperature at which 50% of the original activity remains after heating for 20 min was 46.5± 0.5°C for DHAD from strain 332 and 56.0± 0.5°C for the enzyme from the wild-type.

The DHAD from the mutant and wild-type were easily distinguished on the basis

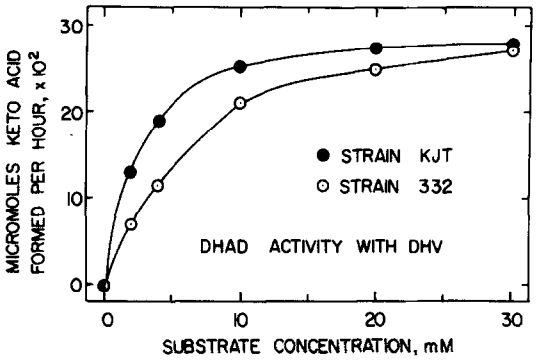

Figure 3. Effect of substrate concentration on production of α-keto acids by preparations of DHAD from Neurospora mutant strain 332 (open circles) and wild-type (closed circles). Fifty and 100 ug protein were used per assay of the wild-type and mutant preparations, respectively.

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of kinetic properties, as shown in figure 3. The K_m values with DHV as substrate were 8.5×10^{-3} M and 3.7×10^{-3} M for the mutant and wild-type preparations, respectively. With the assay method used in this study it was impossible to determine the reaction velocity under conditions in which the substrate concentration was not decreased significantly during incubation. Therefore, the K_m values reported here are not the true Michaelis constants, but nevertheless they are adequate for comparison of the mutant and wild-type enzyme preparations.

DISCUSSION

This study shows clearly that the preparations of DHAD from the iv-1 mutant, strain 332, and the wild-type differ with respect to thermolability, pH-dependence, and kinetics. This can be taken as indirect evidence that the enzymes are also structurally different.

The following model is proposed to account for the deficiency of DHAD in the mitochondria of the iv-1 mutants. It is based upon the present study and several other documented observations (5,6,15-17). (i) DHAD is synthesized on cytoplasmic ribosomes and normally is incorporated into the mitochondria. (ii) The incorporation process depends

partially upon the structural integrity of one or more sites on the DHAD molecule. Such sites have been referred to as "locational-speciticity sites" (18,19). (iii) An alteration in the structure of DHAD caused by mutation at the iv-1 locus can alter the "locational-specificity site(s)" and reduce the affinity between DHAD and the mitochondrial components to which DHAD normally binds. This causes a block in the incorporation process.

Conceivably the hypothetical incorporation process could involve a large number of discrete steps any of which could be blocked by a mutation affecting the structure of a particular "locational-specificity site" on the DHAD molecule. This could result in blocks at any point between the site of synthesis and the site(s) of localization in the mitochondria. In the case of mutant strain 332 the block in the incorporation process may exist somewhere between the outer surface of the mitochondrion and the inner membrane or matrix where DHAD normally is localized (20). The evidence for this is the recent demonstration (21) that DHAD from mutant strain 332 has reduced affinity in vitro for mitochondria previously depleted of DHAD. Of course the presumed structural alteration that reduces the affinity between DHAD from strain 332 and the mitochondria could possibly also reduce the binding between the DHAD and the hypothetical cellular component(s) involved in transporting DHAD from its site of synthesis to the outer surface of the mitochondria. Thus far we have been unable to test this possibility.

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References

1. R. P. Wagner, A. Bergquist, B. Brotzman, E. A. Eakin, C. H. Clarke, and R. N. LePage, in: Organizational Biosynthesis (eds. H. J. Vogel, J. O. Lampen, and V. Bryson) Academic Press, New York, p. 267 (1967).
2. R. P. Wagner, A. Bergquist, T. Barbee, and K. Kiritani, Genetics 49, 865 (1964).
3. D. H. Altmiller and R. P. Wagner, Biochem. Genet. 4, 243 (1970).
4. D. H. Altmiller and R. P. Wagner, Arch. Biochem. Biophys. 138, 160 (1970).
5. D. H. Altmiller, Genetics 71, s2 (1972).

6. D. H. Altmiller, manuscript in preparation.
7. M. Ashwell and T. S. Work, in: Ann. Rev. Biochem. 39, Annual Reviews, Inc., Palo Alto, Calif., p. 251 (1970).
8. N. K. Boardman, A. W. Linnane, and R. M. Smille, eds, Autonomy and Biogenesis of Mitochondria and Chloroplasts, American Elsevier Publishing Company, Inc., New York (1971).
9. R. P. Wagner and A. Berquist, Proc. Nat. Acad. Sci. U. S. 49, 892 (1963).
10. H. J. Vogel, Microbiol. Genet. Bull. 13, 42 (1956).
11. O. H. Lowry, J. H. Rosebrough, A. L. Farr, and R. J. Randall, J. Biol. Chem. 193, 265 (1951).
12. K. Kiritani, S. Narise, and R. P. Wagner, J. Biol. Chem. 241, 2042 (1966).
13. Applications Research Technical Report No. 542 (part no. 566623), Beckman Instruments, Inc., Palo Alto, Calif.
14. Circular Letter ECCL 0172A2, The London Company, Cleveland, Ohio.
15. K. D. Munkres, K. Benveniste, J. Gorski, and C. A. Zuiches, Proc. Nat. Acad. Sci. U. S. 67, 263 (1970).
16. W. A. Scott and H. K. Mitchell, Biochem. 8, 4282 (1969).
17. C. O. Weeks and S. R. Gross, Biochem. Genet. 5, 505 (1971).
18. P. Mitchell, in: Ann. Rev. Microbiol. 13, Annual Reviews, Inc., Palo Alto, Calif. p. 407 (1959).
19. K. D. Munkres and D. O. Woodward, Proc. Nat. Acad. Sci. U. S. 55, 1217 (1966).
20. W. E. Cassady, E. H. Leiter, A. Bergquist, and R. P. Wagner, J. Cell Biol. 53, 66 (1972).
21. D. H. Altmiller, Fed. Proc. 31, 833 Abs. (1972).