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Effect of Mutations in the *qa* Gene Cluster of *Neurospora crassa* on the Enzyme Catabolic Dehydroquinase

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Catabolic dehydroquinase, which functions in the inducible quinic acid catabolic pathway of *Neurospora crassa*, has been purified from wild type (74-A) and three mutants in the *qa* gene cluster. The mutant strains were: 105^{c}, a temperature-sensitive constitutive mutant in the *qa-1* regulatory locus; M-16, a *qa-3* mutant deficient in quinate dehydrogenase activity; and 237, a leaky *qa-2* mutant which posesses very low levels of catabolic dehydroquinase activity. The enzymes purified from strains 74-A, 105^{c}, and M-16 are identical with respect to behavior during purification, specific activity, electrophoretic behavior, stability, molecular weight, subunit structure, immunological cross-reactivity, and amino acid content. The mutant enzyme from strain 237 is 1,500-fold less active and appears to have a slightly different amino acid content. It is identical by a number of the other criteria listed above and is presumed to be a mutant at or near the enzyme active site. These data demonstrate that the *qa-1* gene product is not involved in the posttranslational expression of enzyme activity. The biochemical identity of catabolic dehydroquinase isolated from strains 105^{c} and M-16 with that from wild type also demonstrates that neither the inducer, quinic acid, nor other enzymes encoded in the *qa* gene cluster are necessary for the expression of activity. Therefore the combined genetic and biochemical data on the *qa* system continue to support the hypothesis that the *qa-1* regulatory protein acts as a positive initiator of *qa* enzyme synthesis.

In *Neurospora crassa* quinic acid catabolism is controlled by a tightly linked cluster of four genes, the *qa* cluster (5). Three of these genes are the structural genes for the *qa* enzymes. *qa-2* encodes catabolic dehydroquinase (5-dehydroquinate hydrolyase, EC 4.2.1.10); *qa-3* encodes quinate dehydrogenase (quinate:NAD^{+} oxidoreductase, EC 1.1.1.24); and *qa-4* encodes dehydroshikimate dehydrase (Fig. 1). The fourth gene, *qa-1*, is a regulatory locus whose product controls the expression of the three structural genes. Present evidence suggests that the regulatory protein encoded by the *qa-1* gene acts positively in conjunction with the inducer, quinic acid, to initiate enzyme synthesis (5). An alternate hypothesis is that the *qa-1* gene produces a subunit common to the three enzymes which is necessary for the expression of enzyme activity. In order to distinguish between these two hypotheses, an extensive biochemical comparison of one of the catabolic enzymes isolated from wild-type and three mutant strains has been undertaken. The rationale for this approach is that if the *qa-1* gene product is a structural component of the enzyme, a mutation which affects regulation should also produce an enzyme with altered physical properties.

The enzyme chosen for study was catabolic dehyroquinase, which has previously been purified and thoroughly characterized (6). The four strains used were wild type (74-A), a *qa-3* mutant (M-16), a leaky *qa-2* mutant (237), and a temperature-sensitive constitutive (105^{c}). The *qa-3* mutant, which lacks quinate dehydrogenase activity, does not metabolize quinic acid, and the resulting high intracellular inducer concentration yields levels of induction two to three times higher than wild type. This strain was included in the comparison to verify that mutations in one structural gene do not affect the gene expression or gene product of the other structural loci. Strain 237, although it is a mutant in the structural gene for catabolic dehydroquinase, retains sufficient activity to allow purification of the mutant enzyme. It was included in this study to show that it is possible to detect minor mutational alterations in enzyme structure. Strain 105^{c}, the regulatory mutant used, is noninducible when grown at 25 C, but produces enzyme constitutively when grown at 35 C. Samples of catabolic dehy-

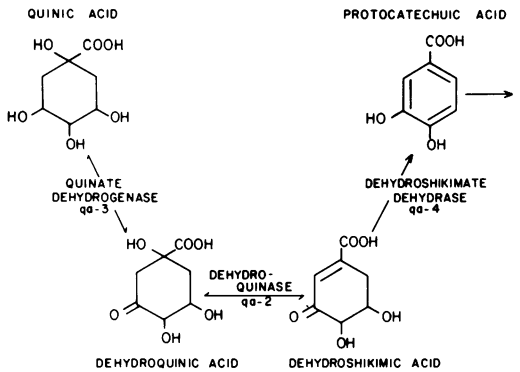

FIG. 1. *Inducible quinic acid catabolic pathway in N. crassa.*

droquinase isolated from the four strains have been compared with respect to behavior during purification, specific activity, electrophoretic behavior, stability, molecular weight, subunit structure, immunological cross-reactivity, and amino acid content.

MATERIALS AND METHODS

Strains. The wild-type strain used was 74-A. The *qa-3* mutant, strain M-16, was derived directly from 74-A (2). The *qa-2* mutant, strain 237, and the temperature-sensitive constitutive mutant, strain 105^c, were isolated from an *arom-9* mutant derived from 74-A (H. W. Rines, Ph.D. thesis, Yale University, New Haven, Conn., 1969).

Growth conditions. Strains 74-A, 237, and M-16 were grown in 200-liter cultures under inducing conditions as described previously (6). Strain 105^c was grown from a conidial inoculum in 2,000-ml Erlenmeyer flasks containing 500 ml of Fries minimal media plus 1.5% sucrose. The cultures were agitated on a rotary shaker at 300 rpm for 24 h at 35 C.

Reagents. Urea, guanidine hydrochloride, and sucrose were Ultra-Pure grade from Schwarz/Mann. Sodium dodecyl sulfate was Sequanol grade from Pierce Chemical Co. Hydrochloric acid for amino acid hydrolyses was Aristar grade from British Drug Houses. Acrylamide monomer was purchased from Eastman and *N,N'*-methylene bisacrylamide monomer from Canalco. All other reagents were reagent grade.

Buffers. The following buffers were employed: (i) 0.1 M potassium phosphate, pH 7.5, plus 0.4 mM dithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride, and 0.1 mM ethylenediaminetetraacetic acid (buffer A); (ii) 0.01 M potassium phosphate (monobasic) plus 0.05 M sodium citrate, pH 3.0 (buffer B); (iii) 0.01 M tris(hydroxymethyl)aminomethane-hydrochloride, pH 8.0, plus 1% sodium dodecyl sulfate, 1 mM ethylenediaminetetraacetic acid and 40 mM dithiothreitol (buffer C; and (iv) 0.1 M tris(hydroxymethyl)aminomethane-0.2 M sodium acetate, pH 7.4, plus 1% sodium dodecyl sulfate and 0.02 M ethylenediaminetetraacetic acid (buffer D).

Enzyme assay. The assay employed for catabolic dehydroquinase has been reported previously (1). Specific activity is expressed as nanomoles of dehydroshikimate produced per minute per milligram of protein at 37 C.

Purification of catabolic dehydroquinase. Catabolic dehydroquinase was purified according to the method described previously (6).

Protein determination. Protein concentrations were determined by the microbiuret technique (7).

Polyacrylamide gel electrophoresis. Analytical polyacrylamide disc gel electrophoresis was performed according to the method of Davis (3). Polyacrylamide disc gel electrophoresis in the presence of sodium dodecyl sulfate was performed according to the method of Fairbanks and co-workers (4). Their technique was modified in that the samples were labeled with the fluorescent dye fluorescamine and then boiled for 15 h prior to electrophoresis. After electrophoresis the protein bands were detected by their fluorescamine fluorescence.

Sucrose density gradient centrifugation. Sucrose density gradient centrifugations were performed according to the method of Martin and Ames (9) at 37,000 rpm and 4 C for 18 h. Catalase (240,000) and alkaline phosphatase (86,000) were used as internal standard proteins for the determination of the native molecular weight; cytochrome *c* (12,400) was used in the subunit molecular weight measurements.

Analytical ultracentrifugation. Sedimentation velocity analyses were done at 52,000 rpm and 4 C on a Beckman model E ultracentrifuge equipped with Schlieren optics according to the method of Schachman (12).

Determination of Stokes radius. The Stokes radius of catabolic dehydroquinase was determined by gel filtration on a Sephadex G-200 column equilibrated with buffer A and calibrated with several standard proteins of known Stokes radius (11).

Immunology. Antisera against catabolic dehydroquinase was produced in New Zealand white rabbits by standard injection, bleeding, and serum separation techniques. Immunological cross-reactivity was assayed by the Ouchterlony double immunodiffusion technique (10) and by the Mancini single radial diffusion method (8).

Amino acid analyses. Amino acid analyses were performed on samples hydrolyzed in vacuo with 6 M hydrochloric acid at 105 C for 24 h. The hydrolysates were analyzed on a Beckman 102-C analyzer.

RESULTS

Purification. Catabolic dehydroquinase was isolated from the four strains with the procedure described previously (6). The behavior of the enzyme during purification was the same for all four strains. All protein preparations were homogeneous as determined by polyacrylamide gel electrophoresis. The overall recovery from strains 74-A, 105^c, and M-16 was 40 to 50%. With strain 237, 20% of the original activity was recovered although this number is subject to a

large error due to the extremely low levels of activity present in this strain. The enzyme isolated from the three high-activity strains had a specific activity of approximately 250, a value identical to that previously reported for pure catabolic dehydroquinase (6). The enzyme from strain 237 was 1,500-fold less active with a specific activity of 0.17.

Catabolic dehydroquinase from the four strains behaved identically on diethylaminoethyl (DEAE)-cellulose chromatography (Fig. 2). Three peaks of activity were observed with strains 74-A, M-16, and 105^{c}. Due to the low level of activity present, only two peaks of activity could be detected for strain 237. Disc gel electrophoresis demonstrated that the three activity peaks each contained a unique electrophoretic species whose electrophoretic mobility correlates with the degree of binding to DEAE-cellulose. These differences in DEAE-binding and electrophoretic mobility have been shown to be the result of slight charge differences in the molecule rather than from major alterations in the enzyme structure (6).

Enzyme stability. Since catabolic dehydroquinase from strain M-16 has been shown to be stable under a variety of denaturing conditions (6), the stability of the enzyme from the various strains was compared. Catabolic dehydroquinase isolated from all four strains is remarkably heat stable and could be incubated at 80 C for 1 h with 100% retention of original activity. The enzyme samples isolated from strains 74-A, M-16, and 105^{c} show identical patterns of reversible dissociation and inactivation at acidic pH. When samples dialyzed into buffer B, pH 3.0, were assayed in the standard pH 7.2 assay mixture, an exponentially increasing activity was observed. Dialysis back into buffer A, pH 7.5, restored full activity.

The stability of catabolic dehydroquinase in the presence of denaturing agents was examined by dialyzing enzyme samples into buffer A plus either 8 M urea or 6 M guanidine hydrochloride. After dialysis into 8 M urea for 18 h at 25 C, enzyme samples from strains 74-A, M-16, and 105^{c} retained 100% of their original activity. Removal of the urea by dialysis back into buffer A did not affect activity. After enzyme samples from the same three strains were dialyzed into 6 M guanidine hydrochloride for 18 h at 4 C, a 75% reduction in activity was observed. Assays of these samples were characterized by an exponential increase in activity which probably results from a time-dependent reactivation after dilution of the guanidine hydrochloride by the assay mixture. Removal of the guanidine hydrochloride by dialysis resulted in recovery of 100% of the original activity.

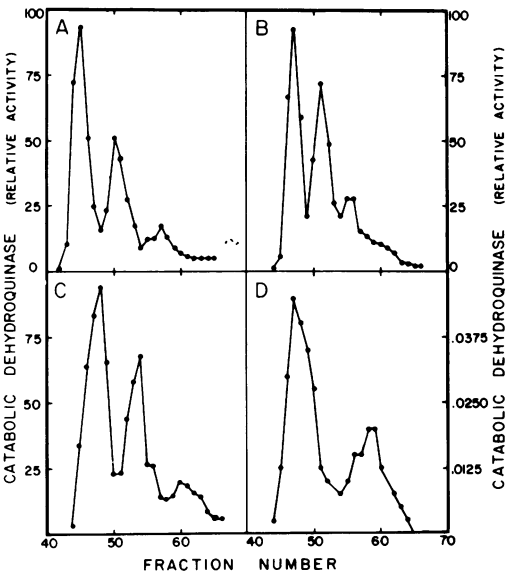

FIG. 2. *DEAE-cellulose chromatography of catabolic dehydroquinase isolated from four strains of N. crassa. As the final purification step, catabolic dehydroquinase in buffer A was bound to a DEAE-cellulose column (2.5 by 35 cm) and eluted with a 1,000-ml, 0.0 to 0.15 M potassium chloride gradient. Panel A, wild-type 74-A; Panel B, qa-3 mutant M-16; Panel C, temperature-sensitive constitutive mutant 105^{c}; Panel D, leaky qa-2 mutant 237.*

Unfortunately the amount of active catabolic dehydroquinase available from strain 237 was insufficient to permit any stability studies except the heat treatment.

Determination of molecular structure. Sucrose density gradient centrifugation of the catabolic dehydroquinase from strains 74-A, M-16, and 105^{c} gave identical values of 167,000 for the apparent native molecular weight. The Stokes radius of the native enzyme from strains 74-A, M-16, and 105^{c} was determined by gel filtration to be 63, 67, and 62 A, respectively. The value previously reported for the enzyme from strain M-16 was 62 A (6). Sedimentation coefficients were determined by sedimentation velocity analyses at 4 C and 52,000 rpm. The values for s_{obs} obtained were 6.8 (74-A), 6.6 (M-16), and 6.1 (105^{c}). The observed Stokes radii and sedimentation coefficients (corrected to correspond to $s_{20,w}$) were used to calculate values for the native molecular weight according to the equations of Siegel and Monty (13). The values obtained were 233,000 (74-A), 241,000 (M-16), and 204,500 (105^{c}).

It was not possible to determine the Stokes radius or sedimentation coefficient for the enzyme from strain 237 due to the limited protein available. However, the elution profile on Sephadex G-200 and the electrophoretic mobility on polyacrylamide gels of this enzyme are similar to those obtained with enzyme samples from the other three strains. This suggests that the structure of the catabolic dehydroquinase isolated from strain 237 is similar to if not identical to that of the wild-type enzyme.

The subunit composition of catabolic dehydroquinase from the four strains under study was compared by polyacrylamide disc gel electrophoresis in the presence of sodium dodecyl sulfate. Enzyme samples from all four strains gave two electrophoretic bands with average molecular weights of 11,300 ± 600 and 23,200 ± 550 (Fig. 3). These appear to be the monomeric subunit and its dimer.

Verification of a 10,000-molecular weight monomeric subunit for catabolic dehydroquinase from strains 74-A, M-16, and 105^{c} was obtained by studies involving the reversible dissociation of the enzyme of acidic pH. Enzyme samples from the three strains were subjected to sucrose density gradient centrifugation at pH 3.0 (buffer B). The three samples gave virtually identical activity peaks (assayed at pH 7.2) corresponding to a molecular weight of approximately 10,000 (Fig. 4A). The active fractions from each of the gradients were combined, dialyzed into buffer A (pH 7.5) and recentrifuged in sucrose density gradients in buffer A. The activity from all three strains returned to the position of the native enzyme (Fig. 4B).

Immunological comparisons. Antisera against catabolic dehydroquinase were prepared with pure enzyme from strain M-16. Double diffusion analysis on Ouchterlony plates demonstrated that the enzymes from all four strains are immunologically identical with both native enzyme and subunit precipitin lines present. Single diffusion analysis on Mancini plates showed that the diameter of the precipitin ring around the antigen well is correlated with the measured protein concentration of each sample. Strain 237, which has very low enzyme activity, is immunologically competent with wild-type levels of catabolic dehydroquinase protein.

Amino acid analysis. The amino acid content of strains 74-A, M-16, and 105^{c} are identi-

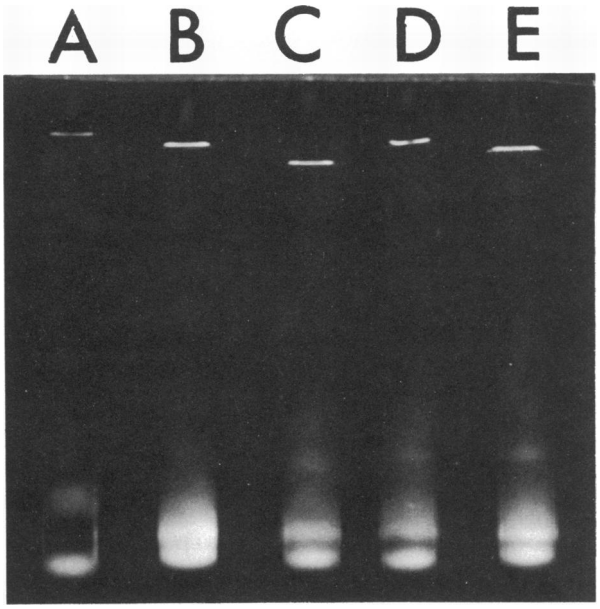

FIG. 3. *Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of catabolic dehydroquinase isolated from four strains of N. crassa. The protein samples were labeled with the fluorescent dye fluorescamine and then boiled for 15 h in buffer C. The 5.6% polyacrylamide gels were prepared and run in buffer D. The fluorescent gels were photographed using Polaroid type 57 film (ASA 3,000) with a no. 16 Wratten gelatin filter. Gel A, cytochrome c (molecular weight 12,400); gel B, wild-type 74-A; gel C, temperature-sensitive constitutive mutant 105^{c}; gel D, qa-3 mutant M-16; and gel E, leaky qa-2 mutant 237. The leading band in all gels is the marker dye pyronin Y. The two bands on the catabolic dehydroquinase gels correspond to molecular weights of 11,300 and 23,200.*

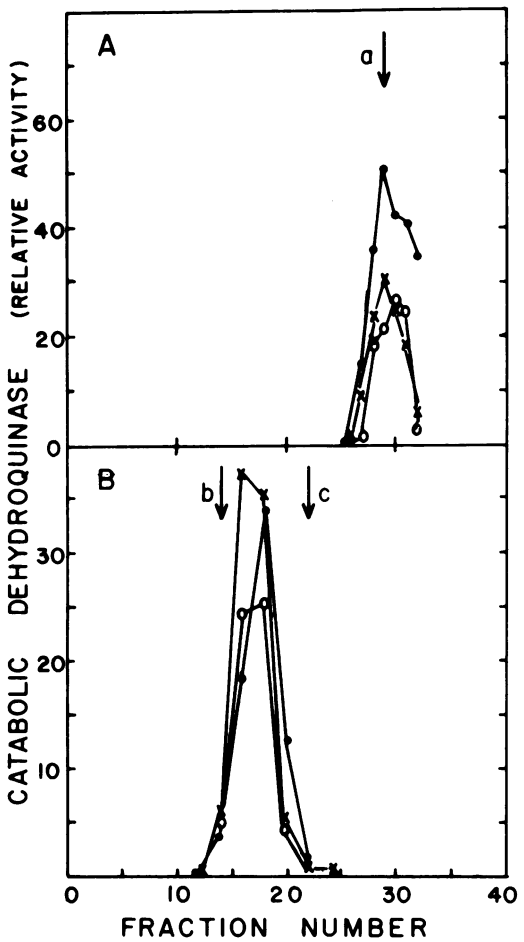

FIG. 4. *Sucrose density gradient centrifugation of catabolic dehydroquinase isolated from three strains of N. crassa: wild-type 74-A* (○); *qa-3 mutant M-16* (●); *and temperature-sensitive constitutive mutant 105^c* (×). *Panel A, the enzyme samples and the gradients were in buffer B, pH 3.0, with cytochrome c* (*a*) *as an internal standard. Panel B, active fractions from gradients in Panel A were combined, dialyzed into buffer A, pH 7.5, and recentrifuged on gradients in buffer A with catalase* (*b*) *and alkaline phosphatase* (*c*) *as internal standards. Fractions from all gradients were assayed in the standard pH 7.2 assay buffer.*

cal within the limits of experimental error (Table 1). For these analyses enzyme samples from each strain were prepared in duplicate, hydrolyzed at the same time, and analyzed sequentially on a single amino acid analyzer. These data are comparable to the numerous analyses of catabolic dehydroquinase performed previously. Data not included in Table 1 demonstrated that the low value for proline in strain 74-A is an artifact. The amino acid content of strain 237, which is presumed to be a point mutation in the *qa-2* structural gene, is also very similar (Table 1). There is an indication of an increase of one histidine residue and a possible decrease of either one valine or one isoleucine residue. More extensive analysis will be necessary to determine the exact amino acid changes that have taken place in this strain.

DISCUSSION

The biochemical comparison of catabolic dehydroquinase isolated from wild-type 74-A and the regulatory mutant 105^c demonstrates that the enzyme from the two sources is identical. Thus there appears to be no posttranslational interaction of the product of the regulatory gene and the structural gene products. Having eliminated this possibility, the available genetic and biochemical data continue to support the hypothesis that the regulatory gene, *qa-1*, encodes a regulatory protein which in conjunction with the inducer, quinic acid, acts as a positive initiator of protein synthesis (5). The enzyme from strain 105^c which is synthesized constitutively, i.e., in the absence of inducer, appears identical to the enzyme from induced strains by the properties compared. Therefore, we conclude that the inducer, quinic acid, plays no role in posttranslational assembly and/or activation of the native enzyme.

The biochemical identity of enzyme isolated from wild type and the constitutive strain with that from the *qa-3* mutant, strain M-16, indicates that there is no mandatory interaction of the individual structural *qa* gene products.

TABLE 1. *Amino acid composition of catabolic dehydroquinase from four strains of Neurospora crassa*

Amino acid	μmol%			
	74-A[a]	M-16	105^c	237
Lysine	2.3	2.5	2.1	2.8
Histidine	5.1	4.6	4.2	5.7
Arginine	4.8	4.5	4.2	5.0
Aspartic acid	7.6	8.0	8.0	7.6
Threonine	6.9	6.8	7.5	6.9
Serine	9.3	9.4	10.0	9.7
Glutamic acid	8.9	8.8	8.2	8.2
Proline	7.1	8.6	7.8	8.4
Glycine	10.4	10.2	10.6	10.3
Alanine	10.8	10.7	11.4	10.8
Valine	4.2	4.2	4.2	3.7
Methionine	1.1	0.8	0.8	0.7
Isoleucine	5.5	5.2	5.2	4.7
Leucine	10.0	9.8	9.8	9.6
Tyrosine	2.1	2.1	2.3	2.1
Phenylalanine	3.9	3.6	4.0	3.8

[a] Strain.

Although each *qa* enzyme appears to act independently of the other, this does not rule out a spatial organization or intracellular localization.

Catabolic dehydroquinase isolated from the *qa-2* structural gene mutant, strain 237, differed from the enzyme of the other strains by two criteria. The specific activity is 1,500-fold lower than the wild-type enzyme. There is also an apparent alteration in amino acid content. These data are indicative of a mutation at or near the catalytic site of the enzyme which has little or no influence on the native structure of the molecule. It is not known whether the alteration in catalytic activity results from a reduction in substrate affinity or a reduction in substrate conversion.

ACKNOWLEDGMENTS

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