MITOCHONDRIAL MALATE DEHYDROGENASE: FURTHER STUDIES ON MULTIPLE ELECTROPHORETIC FORMS

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SUMMARY

Previous studies on chicken heart mitochondrial malate dehydrogenase suggested that the multiple electrophoretic forms differed only in conformation. Further experiments have now shown that, while it is possible to effect marked changes in the electrophoretic mobility of the various forms as previously described by extended reversible denaturation in either acid media or concentrated guanidine hydrochloride, the products of these treatments differ from the native enzyme in thermal stability.

Mitochondrial malate dehydrogenases from a number of species have been shown, by electrophoretic techniques, to exist in several enzymatically active forms (1). Initial studies by Thorne, Grossman and Kaplan (1) with porcine mitochondrial malate dehydrogenase (MDH) indicated that the multiple electrophoretic forms differed little in their catalytic properties. Further experiments (2) confirmed the lack of significant catalytic differences between the electrophoretic forms and indicated also that they possessed similar resistance to thermal inactivation. That the multiple forms were not the result of aggregation phenomena was evidenced by the apparent homogeneity in ultracentrifugal studies of samples containing several electrophoretic species and by the elution of such samples in a symmetrical peak in gel filtration experi-

ments with Sephadex G100 and G-200. Amino acid analysis of the separate electrophoretic forms failed to reveal any significant differences in composition. Further, analysis of peptide maps in both our own and other laboratories suggested that the mitochondrial malate dehydrogenases were composed of two very similar, if not identical subunits (3). Differences were noted, however, in the susceptibility of the individual electrophoretic components to inactivation upon treatment with iodine and para-hydroxymercuribenzoate (2). The above experiments suggested that the electrophoretic forms might differ in conformation, and optical rotatory dispersion studies (4) indicated that this was indeed the case. A consideration of the above findings led us to postulate that the multiple electrophoretic forms did not differ in primary structure but resulted from differences in conformation. We suggested that the term "conformers" be applied to such enzyme systems.

We felt, for reasons treated in detail in a paper by Epstein and Schechter (5), that reversible denaturation studies would provide a useful test of the conformer hypothesis. In our hands, reversible acid denaturation of the anodally migrating forms of chicken heart mitochondrial MDH led to the conversion of these forms to species with the same electrophoretic mobility as those of the cathodal forms of the untreated enzyme. Such results were in agreement with the "conformer" hypothesis. More recently, Schechter and Epstein (6) have carried out comparable reversible denaturation experiments, but in this case using guanidine hydrochloride as the denaturing agent. These workers were not able to confirm the reported interconversion of electrophoretic forms. In order to resolve these conflicting results, we have carried out further reversible denaturation studies on chicken heart mitochondrial MDH, using both acid and guanidine hydrochloride as denaturants. As detailed below, we were able to confirm our observation that reversible denaturation can, under certain conditions, cause marked changes in the electrophoretic mobility of the anodal bands; however, we now report that the products of such treatment differ from the native enzyme in thermal stability.

METHODS

Chicken heart mitochondrial MDH was purified as described previously (7). The enzyme was homogeneous as judged by ultracentrifugal studies. A major difficulty, in both our own and other previous studies, has been the satisfactory resolution of the multiple electrophoretic forms. In our earlier work the enzyme was subjected to chromatography on carboxymethyl cellulose using a very shallow linear salt gradient for elution. Small fractions of the eluate were collected and each of these was examined by starch gel electrophoresis to determine the extent of electrophoretic heterogeneity. Only by discarding more than 99°/o of the total protein were we able to obtain samples with minimal contamination by adjacent electrophoretic bands. In our present studies we were able to achieve significantly better resolution of the electrophoretic forms of chicken heart mitochondrial MDH through the use of isoelectric focusing (8). Satisfactory resolution was obtained with a single run using a pH gradient from 5 to 8 and loadings up to 200 mg of protein. Doubtless even better fractionation could be achieved by rerunning selected samples over a narrower pH range. Samples of the individual electrophoretic forms of mitochondrial MDH, prepared by electrofocusing and selected for minimal electrophoretic heterogeneity, were subjected to the reversible denaturation procedures described below. All samples were dialysed overnight against several changes of distilled water to remove the ampholytes and sucrose introduced in the isoelectric focusing. Concentration of the samples was performed by pressure ultrafiltration using Diaflo UM-10 membrane. Reversible denaturation in acid and guanidine hydrochloride was carried out in essentially the same manner as described previously (4,6,9). For reversible acid denaturation an extensively water dialysed enzyme sample (0.5 mg/ml) was brought to 0.05 M in dithiothreitol and an 0.2 ml aliquot was carefully titrated to pH 2.0 with 0.1N HCL in a polyethylene tube. For reversible denaturation in guanidine hydrochloride dialysed enzyme samples were placed in 7.6 M guanidine hydrochloride made up in 0.1 M citrate pH 7.0 containing 0.05 M dithiothreitol to

give a final enzyme concentration of 0.5 mg/ml. After 10 minutes no activity was detectable in a spectrophotometric assay (1) in either the acid or guanidine hydrochloride treated samples. Samples were removed at intervals over a twenty-four hour period and renaturation was initiated by diluting 20-fold with 0.5 M citrate pH 7.0 containing 0.05 M dithiothreitol and 0.5 mg/ml DPNH. Maximal recovery of enzymatic activity was attained approximately 2 hours after dilution in both cases. Comparable initial activities of enzyme were used in all cases and both denaturation and renaturation were carried out at room temperature. Controls were carried through the same procedures with the omission of the denaturing agent.

RESULTS

As had previously been noted by Schechter and Epstein, we found the electrophoretic forms to differ in the degree to which they could be renatured after guanidine hydrochloride treatment, the more cathodal bands being recovered in higher yield than the anodal forms. Similar results were obtained with reversible acid denaturation although in contrast to the experience of Schechter and Epstein we were able to obtain significant renaturation (up to 30%) with the anodal bands. It is possible that the difference in results of Schechter and Epstein and ours were that their preparations contained some impurities as well as their using lower levels of MDH in their studies. This may account for the considerably smaller percentage of recovery of active enzyme than was achieved in our renaturation experiments. The reversibly denatured samples, along with the respective controls were examined by starch gel electrophoresis at pH 7.0 using a specific stain for MDH activity (1).

Renatured samples of the original anodal forms (Bands D and E) which had been subjected to prolonged denaturation (12 to 24 hours) in either guanidine hydrochloride or acid were found to be converted to forms with electrophoretic mobilities comparable to the more cathodal bands of the native enzyme (Fig. 1). No significant change in mobility was noted upon reversible denaturation of the most cathodal form (Band A). These findings are essentially the same as

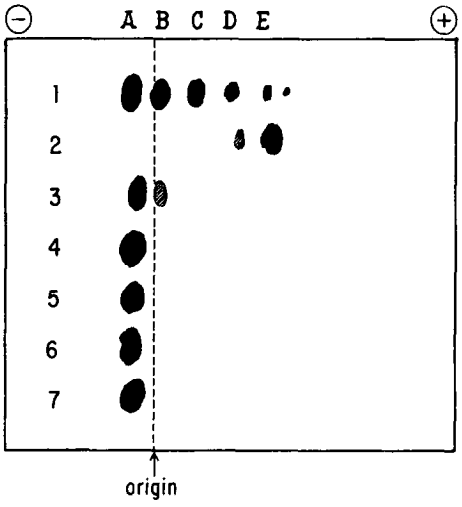

Figure 1. Starch gel electrophoresis of chicken mitochondrial MDH subjected to reversible acid and guanidine hydrochloride denaturation and stained for enzyme activity. (1) unfractionated enzyme; (2) untreated Band E; (3) untreated Band A; (4) Band E reversibly denatured in acid; (5) Band A reversibly denatured in acid; (6) Band E reversibly denatured in guanidine hydrochloride; (7) Band A reversibly denatured in guanidine hydrochloride. Samples (4) through (7) were treated with denaturant for 24 hours.

reported earlier (4). When samples of the anodal bands which had been treated with denaturant for considerably shorter periods (30 minutes or less) were examined after renaturation, considerably less conversion of electrophoretic forms was noted. This was particularly true of those samples treated with guanidine hydrochloride.

In our earlier studies we had interpreted the changed mobility of the anodal electrophoretic form to a more cathodal form after reversible acid denaturation as a true interconversion from one native form to another. However, as pointed out by Schechter and Epstein, electrophoretic data is at best semiquantitative. We therefore carried out studies on the thermal stability of the original cathodal form of MDH (Band A) and the cathodal forms of MDH generated by acid and guanidine hydrochloride reversible denaturation of the original Band A and anodal bands (Bands D and E) of MDH. As illustrated in Fig. 2 we found the products of prolonged acid and guanidine hydrochloride

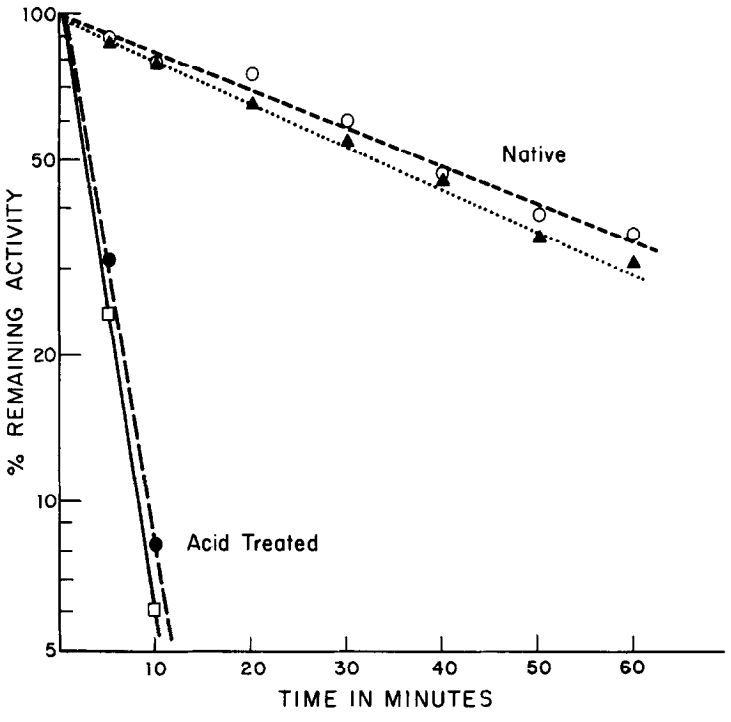

Figure 2. Heat stability of native and reversibly acid denatured forms of chicken mitochondrial MDH. Samples were heated at 50° C and aliquots were removed at intervals, chilled immediately to 0° C and later assayed; -O- untreated Band A; -▲- untreated Band E; -●- reversibly acid denatured Band A; -□- reversibly acid denatured Band E.

treatment of both the A Band and the anodal bands to be very substantially less heat resistant than the original Band A. The products from both the cathodal and anodal bands appear to be identical in their heat stabilities but distinct from the original Band A.

The enzyme with altered electrophoretic mobility observed in the present studies is probably related to the altered electrophoretic forms noted after prolonged dialysis against saturated ammonium sulfate (4, footnote 16) or prolonged storage at 4° C (6, footnote 16).

It should be noted that in early studies (9) on the reversible denaturation of unfractionated chicken heart mitochondrial MDH, where we reported that enzyme reversibly denatured in guanidine hydrochloride had immunological, electrophoretic and kinetic properties indistinguishable from the native

enzyme, only short periods of exposure to denaturing conditions (less than 10 minutes) was employed.

Our results, in contrast to that of Schechter and Epstein, indicate that marked changes in electrophoretic mobility can be achieved with retention of at least partial catalytic activity. The altered properties of the products obtained after extensive treatment with denaturants reveal, however, that a true interconversion of electrophoretic forms is not obtained under these conditions. However, the fact that bands E and A after denaturating can be converted to some common form, supports the general view of interconversion of enzyme forms. Interconversion of forms have also been recently observed with purified preparations of horse liver alcohol dehydrogenase (9).

We do not believe that our present results negate the "conformer" hypothesis which was based on a variety of different types of evidence of which electrophoretic interconversion was only a part. Aspartate-glutamic transaminase appears to exist in different conformers which are not interconvertible after dissociation and reassociation (10, 11). As detailed by several investigators (12, 13) the rates and extent of renaturation of a number of enzymes are markedly affected by such parameters as pH, temperature, salt and protein concentration, and the discrepancies between our results and those of Schechter and Epstein may simply lie in variations in experimental procedure.

Studies on rat liver mitochondrial MDH have suggested an alternative explanation for the presence of multiple electrophoretic forms (14); however, the possibility that the rat mitochondrial MDH multiple forms may be due to allelic forms of the enzyme has not been excluded. There is no evidence for such allelic forms in the chicken preparations (15). Further, the recent report by Thorne (16) of the interconversion of rat brain MDH electrophoretic forms with full retention of activity renders the hypothesis of Mann and Vestling less tenable. We believe that an unequivocal basis will finally depend on detailed structural studies of the resolved enzyme forms. Such

studies are currently being carried out in both our own and other laboratories.

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