Kinetics and Electrophoretic Properties of the Isoenzymes of Aspartate Aminotransferase from Pig Heart*

J. S. NISSELBAUM AND OSCAR BODANSKY

From the Division of Biochemistry, Sloan-Kettering Institute for Cancer Research, Department of Biochemistry, Memorial Hospital, and Sloan-Kettering Division of Cornell University Graduate School of Medical Sciences, New York, New York 10021

SUMMARY

Cationic aspartate aminotransferase from pig heart was completely destroyed by heating to 75° for 20 min in the purification procedure of Jenkins, Yphantis, and Sizer (11). Chromatography of the partially purified enzyme preparation on carboxymethyl cellulose according to the method of Henson and Cleland (8) yielded two enzyme fractions both of which migrated toward the anode upon starch gel electrophoresis. These two fractions were also kinetically and immunochemically indistinguishable from each other. Pig heart mitochondrial aspartate aminotransferase migrated toward the cathode upon starch gel electrophoresis. It differed both kinetically and immunochemically from the anionic isozyme of pig heart.

In view of this discrepancy, it was decided to investigate the electrophoretic and immunochemical as well as the kinetic properties of the preparations of pig heart aspartate aminotransferase obtained by the method of Henson and Cleland (8) and designated by them as cationic and anionic isozymes. In addition, the mitochondrial fraction of this enzyme was isolated by centrifugation, and its kinetic, electrophoretic, and immunochemical properties were determined.

EXPERIMENTAL PROCEDURE

Enzyme Assay—Aspartate aminotransferase activity was determined by the method of Karmen (9); L-aspartic acid (Cycle Chemical) was used instead of DL-aspartic acid. Enzyme was diluted in 0.157, human serum albumin (Cutter) buffered with 0.033 M phosphate buffer, pH 7.4. The units of activity have been previously defined (5).

Starch Gel Electrophoresis—The electrophoretic characteristics of aspartate aminotransferase in homogenates and in purified fractions of pig heart were determined in starch gels. The gels were prepared as previously described (10) but were made up in 5.0 mM Tris-succinate buffer, pH 7.2. The final pH of the gels was 6.8 to 7.0. The electrode vessels contained 0.1 M phosphate buffer, pH 7.2. Electrophoresis was carried out in a cold room (4-6°) for 18 hours at 4.0 volts per cm and 10 to 16 ma. The gels were sliced and stained specifically for aspartate aminotransferase by the method of Schwartz, Nisselbaum, and Bodansky (10).

RESULTS

Purification of Aspartate Aminotransferase Isozymes from Pig Heart—Pig heart aspartate aminotransferase was prepared as described by Henson and Cleland (8) who followed the procedure of Jenkins, Yphantis, and Sizer (11). Fresh pig heart ventricle was homogenized, heated at 75° for 20 min in maleate-EDTA buffer in the presence of α-ketoglutarate, and fractionated with ammonium sulfate as described by Jenkins, Yphantis, and Sizer (11). Neither these investigators nor Henson and Cleland submitted values for the yield of enzyme activity after heating the heart homogenate. In the present study the activity was found to be decreased to 52% and was recovered chiefly in the fraction between 50 and 67% saturation with ammonium sulfate. This fraction was redissolved in potassium maleate buffer, 0.3 M, pH 6.0, and was dialyzed according to the procedure of Henson and

* This work has been supported in part by Grant P-164 from the American Cancer Society and Grant CA 08748 from the National Cancer Institute, National Institutes of Health.
Fig. 1. Elution diagram of partially purified pig heart aspartate aminotransferase from a CM-cellulose column. Each tube collected approximately 4 ml. The first peak emerged with 5 mM sodium acetate buffer, pH 5.4, which had been used to equilibrate the column and the enzyme. A gradient to 100 mM buffer at the same pH was started at tube 26. The tubes that were pooled in the separate fractions are indicated and are referred to in the text as CM-cellulose Peak 1 and Peak 2.

Cleland (8) against three 7-liter changes of 5 mM sodium acetate buffer, pH 5.4. It was then clarified by centrifugation and placed on a column of carboxymethyl cellulose that had been equilibrated previously with the same buffer. Henson and Cleland (8) gave no indications of column dimensions or load of enzyme. In the present study, all of the enzyme solution, containing approximately 250 mg of protein was applied to a column of CM-cellulose (2.0 x 33.0 cm) obtained from the same source, Bio-Rad Laboratories, used by Henson and Cleland (8). The column was then washed with 5 mM sodium acetate buffer, pH 5.4, until the optical density at 280 m\(\mu\) of the effluent approached the optical density of the buffer, i.e. 125 ml. At this point (Fig. 1) 100 ml of 100 mM acetate buffer, pH 5.4, were introduced into 100 ml of 5 mM buffer so as to yield a gradient for elution. Fig. 1 shows that two peaks of protein coinciding with two major peaks of aspartate aminotransferase activity were recovered from the CM-cellulose column. One passed through the column at the original buffer concentration, and one was eluted at the higher buffer concentration. The effluents in each of the two major peaks of enzyme activity were pooled as indicated in Fig. 1 and were concentrated by precipitation at 67% saturated ammonium sulfate. The sum of the enzyme activities of these two pools represented 80% of the enzyme activity placed on the column.

Fig. 2 shows that the homogenate of pig heart in maleate-EDTA buffer contained an anionic and a cationic isozyme of aspartate aminotransferase, that heating this homogenate for 20 min at 75°, even in the presence of added \(\alpha\)-ketoglutarate, destroyed the cationic component, and that the remaining enzyme, when chromatographed on CM-cellulose, yielded two major fractions, both of which were anionic.

Pig heart mitochondria were isolated according to the method of Smith (12), which includes washing three times by suspension in buffered 0.25 M sucrose solution, and sedimenting at 26,000 x g for 15 min. The washed mitochondria were then suspended in 2.0 ml of 0.067 M phosphate buffer, pH 7.4, and disrupted by sonic oscillation at 20 kc in a 60-watt M. S. E. (Instrumentation Associates, Inc., New York) sonic disintegrator for 1 min. The preparation used in this study was Lot B-1460 and had a capacity of 0.54 meq per g.
instrument was tuned to give maximal agitation in the mitochondrial suspension which was kept immersed in ice-cold water. Fig. 2 shows that this preparation contained only the cationic isozyme of aspartate aminotransferase. Since the staining method for aspartate aminotransferase detects 300 units of enzyme per ml (10), the enzyme in the stages after heating could have contained no more than 0.1 to 0.001% of the cationic component. Conversely, the mitochondrial fraction could have contained no more than 0.01% of the anionic component.

**Kinetics**—The $K_m$ (L-aspartate) and the $K_m$ (a-ketoglutarate) of the anionic and cationic isozymes of aspartate aminotransferase from pig heart were determined by the assay method of Karmen (9) and according to the procedure of Velick and Vavra (7) (Table I). The values of $K_m$ (L-aspartate) for the anionic components were very similar to the values of 5.0 mM, 6.4 mM, and 4.3 mM reported for the anionic components of bovine liver (6), human heart (5), and human liver (5), respectively. The values for $K_m$ (a-ketoglutarate) were in the same range as the values of 0.90 mM, 0.73 mM, and 0.60 mM, respectively, reported for the anionic isozymes from the above tissues. $K_m$ (L-aspartate) for the mitochondrial cationic component was similar to the values of 0.40 mM, 0.84 mM, and 1.0 mM obtained, respectively, for the cationic isozymes from beef liver (9), human heart (5), and human liver (5). $K_m$ (a-ketoglutarate) was significantly higher for the mitochondrial cationic component than for the anionic components (Table I), but was somewhat lower than the values of 2.0 mM, 3.1 mM, and 3.3 mM reported for the cationic isozymes from beef liver (6), human heart (5), and human liver (5), respectively.

**Immunochemistry of Pig Heart Aspartate Aminotransferase Preparations**—The $K_m$ values of the anionic isozyme from the heart and liver of the different species studied were in the same range. A similar situation holds for the cationic isozyme from these tissues from different species. Accordingly, it was of interest to determine the extent to which this similarity among the like isozymes from different species was also manifest immunochemically. It had been shown previously (5) that rabbit antiserum to the anionic isozyme from human heart inhibited completely the anionic isozyme from human heart or human liver, but did not react with the cationic isozyme. Conversely, the rabbit antiserum to the cationic isozyme from human heart inhibited completely the homologous isozyme, but did not react with the anionic isozyme. Table II shows that rabbit antiserum to the anionic isozyme from human heart inhibited both anionic preparations from the pig heart to the extent of about 85%, but did not inhibit the cationic isozyme. The rabbit antiserum to the cationic isozyme from human heart did not inhibit the anionic preparations from pig heart and, although the inhibition of the mitochondrial isozyme was not complete, it was none the less substantial.

**DISCUSSION**

Henson and Cleland (8) assumed that when the 50 to 67% saturated ammonium sulfate fraction of pig heart aspartate aminotransferase, prepared according to Jenkins, Yphantis, and Sizer (11), was chromatographed on a CM-cellulose column, elution with 5 mM sodium acetate at pH 5.4 yielded the anionic isozyme, and that further elution with higher concentrations of buffer yielded the cationic isozyme. The electrophoretic characteristics of these two peaks were not presented.

Martinez-Carrion, Riva, Turano, and Fasella (13) have also prepared the enzyme according to the procedure of Jenkins, Yphantis, and Sizer (11) except for the use of succinate instead of maleate buffer. Upon subjecting their preparation to gel electrophoresis at pH 8.0 (14), they obtained three major anionic

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**TABLE I**

Values of $K_m$ for pig heart aspartate aminotransferase preparations

The reaction mixtures contained 0.085 mM NADH, 2000 optical density units of malic dehydrogenase, appropriately diluted enzyme, L-aspartate, a-ketoglutarate, and 0.067 M phosphate buffer, pH 7.4, to give a final volume of 3.0 ml. L-Aspartate concentration varied between 0.2 and 20 mM for the anionic isozyme and between 0.6 and 20 mM for the cationic isozyme. The a-ketoglutarate concentration was varied between 0.2 and 2.0 mM for the anionic isozyme and between 0.8 and 5.0 mM for the cationic isozyme. The reactions were started by addition of (Y-phantis, and Sizer (11) except for the use of succinate instead of maleate buffer. Upon subjecting their preparation to gel electrophoresis at pH 8.0 (14), they obtained three major anionic

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**TABLE II**

Inhibition of aspartate aminotransferase preparations by antisera to anionic and cationic isozymes from human heart

Enzyme solution (1.0 ml) containing approximately 300 units of aspartate aminotransferase was mixed with 1.0 ml of either a 1:5 dilution of antiserum to human heart anionic aspartate aminotransferase or a 1:10 dilution of the antiserum to the human heart cationic enzyme. After incubation at 37° for 2 hours and at 4° for 66 hours, the mixtures were centrifuged and the aspartate aminotransferase activity in 0.5 ml of the clear supernatant was determined. The extent of inhibition was calculated from controls that contained a 1:10 dilution of normal rabbit serum in place of the antiserum. Each value represents the average of two determinations.

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<td>Anti-anionic</td>
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* These values were reported previously (5).
bands of aspartate aminotransferase activity. They did not present any kinetic or immunochemical data concerning these anionic bands. These investigators also indicated that the mitochondria might contain several cationic electrophoretic components. They suggested that, regardless of the electrophoretic characteristics of the multiple forms, Henson and Cleland (8) may have studied kinetically similar components of the various multiple forms of the two isozymes, whereas our study (5) and that of Boyd (4) concerned itself with two kinetically different forms of the isozymes.

The present study shows that heating of the homogenate of pig heart in maleate-EDTA buffer at pH 6.0 at the beginning of the purification procedure resulted in the complete destruction of the mitochondrial cationic isozyme. Wada and Morino (15) also demonstrated that in the presence of α-ketoglutarate and other stabilizers the mitochondrial isozyme of aspartate aminotransferase was more heat labile than the supernatant isozyme.

Chromatography in the present study under the conditions used by Henson and Cleland (8) did indeed result in the isolation of two peaks of aspartate aminotransferase activity. However, both of these peaks were anionic at pH 6.8 to 7.0 by electrophoretic criteria. The $K_m$ (aspartate) and $K_m$ (α-ketoglutarate) values obtained for these two anionic components in the present study were essentially the same as those obtained by Henson and Cleland (8). The cationic isozyme prepared by us from the mitochondria of pig heart yielded values for the Michaelis constants that were greatly different from those for the anionic isozyme and, as has already been noted, were close to the values obtained for the cationic isozymes of aspartate aminotransferase solated from beef liver (6), human heart (5), and human liver (5). The present studies explain the discrepancy between the kinetic results of Henson and Cleland (8) and those of other workers (1-6).

Acknowledgment—The authors wish to acknowledge the technical assistance of Mrs. May B. Gregg.

REFERENCES
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