Mitochondrial Aspartate Transaminase. II. Isolation and Characterization of the Multiple Forms*

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ABSTRACT: Mitochondrial pig heart glutamate–aspartate transaminase (L-aspartate:2-oxoglutarate aminotransferase, EC 2.6.1.1) can be separated by ion-exchange chromatography on carboxymethyl-Sephadex into three fractions each possessing a different electrophoretic mobility. The absorption spectral properties of the fractions have been found to be similar. The subforms are equal in pyridoxal phosphate content, but differ in the relative amounts of "catalytically active" (absorbance at 430 mμ at low pH and 360 mμ at high pH) and "catalytically inactive" (absorbance at 340 mμ at high or low pH) pyridoxal phosphate which accounts for differences in their specific activities and molecular mean residue ellipticity at 430 mμ. The subforms display identical reaction rates with the pseudo-substrates, L-alanine, L-methionine, and L-serine. Their true dissociation constants with α-methylaspartate are equivalent. No distinctions among the multiple forms can be derived from the amino acid composition or peptide maps, pointing out their similar primary structure. Optical rotatory dispersion studies in the ultraviolet region of the spectrum and immunological double diffusion on agar gel cannot distinguish the subforms ruling out the possibility of the mitochondrial glutamate–aspartate transaminase fractions being conformers.

Although the pig heart mitochondrial isozyme is distinct from the supernatant one, a comparison among the subforms of each isozyme system points out many similarities. No variation was perceived in the structure of the proteins within each system of multiple forms. The possibility of minor localized structural or conformational differences, which could not be detected by the procedures used, is speculated.

A broad spectrum of enzymes with varying activities and cellular localizations possess multiple forms or isozymes. Changes in the physical properties of proteins resulting in multiple molecular forms can be artificially produced during the purification procedure as in cytochrome C (Flatmark, 1967; Margoliash and Lustgarten, 1961). In other enzymes, however, multiple forms can be detected from the beginning of the isolation procedure. And in well-characterized isozyme systems such as lactic dehydrogenase (Cohn *et al.*, 1962; Dawson *et al.*, 1967) and fructose diphosphate aldolase (Penhoet *et al.*, 1967) subforms represent hybrids of nonidentical subunits. But, the hybrid theory cannot be used as an explanation for all systems of multiple enzymes. Among other possibilities is the recent usage of the term "conformer" by Kitto *et al.* (1966) to describe those multiple forms of chicken malic dehydrogenase which appeared to differ only in conformation. On the other hand, the electrophoretically distinct forms in another dehydrogenase, alcohol dehydrogenase from *Drosophila melonogaster*, can be interconverted by the removal and addition of NAD as cofactor (Jacobson, 1968). Hence, there is a variety of interpretations of the structural nature of multiple forms of enzymes. Nevertheless, most cases can be explained by: (1) structural differences among the subforms; (2) the binding of different amounts of metal ion or coenzyme to the various forms; (3) variations in the content of carbohydrate or lipid bound to the protein; (4) structural modifications due to the isolation procedure; and (5) a combination of nonidentical subunits in various ratios.

Glutamate–aspartate aminotransferase exists as two different enzymes in dog heart (Fleischer *et al.*, 1960), rat liver (Boyd, 1966), and pig and human heart (Wada and Morino, 1964; Niesselbaum and Bodansky, 1964, 1966). One of these isozymes is associated with the cytoplasmic fraction, supernatant glutamate–aspartate transaminase, while the other is mitochondrial in origin, mitochondrial glutamate–aspartate transaminase. In pig heart (Martinez-Carrion *et al.*, 1965) several subforms are associated with each isozyme. The subforms of supernatant glutamate–aspartate transaminase were isolated and characterized according to their physical, chemical, and functional properties. Apart from electrophoretic mobility the most obvious physical difference among the isolated fractions was the mode of coenzyme binding (Martinez-Carrion *et al.*, 1967). Enzyme multiplicity was also found for the mitochondrial enzyme. A single heart homogenate from a freshly killed pig showed at least two distinct mitochondrial glutamate–aspartate transaminase activity bands moving toward the cathode in starch gel electrophoresis at pH 8.6. Therefore, a study of the properties of the multiple forms of pig heart mitochondrial glutamate–aspartate transaminase was undertaken to investigate the molecular nature of the mitochondrial glutamate–aspartate

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transaminase subforms and gain insight into their biological role and function in comparison with other systems of multiple forms and especially that of supernatant glutamate–aspartate transaminase. The present work describes the separation of the three main subforms of pig heart mitochondrial aspartate aminotransferase and an examination of the structural and kinetic properties of each form.

Experimental Procedure and Results

Materials. DEAE-Sephadex, CM-Sephadex, malate dehydrogenase, NADH, α-ketoglutarate, α-methylaspartate, and fast violet B salt (diazolate 6-benzoylamino-4-methoxy-*m*-toluidine) were obtained from Sigma; cysteinesulfinic acid, trypsin, and amino acids from Calbiochem; *p*-mercuribenzoate from Mann Research Laboratories; and hydrolyzed starch from Connaught Medical Research Laboratories.

Purification and Separation of Mitochondrial Glutamate–Aspartate Transaminase Multiple Forms. Mitochondrial glutamate–aspartate transaminase was isolated and purified essentially by the method of Wada and Morino (1964) utilizing the additional purification steps of Martinez-Carrion and Tiemeier (1967) with the exception of the following. At the beginning of the procedure the heat step was carried out in 0.05 M sodium glutarate buffer (pH 6.9) instead of sodium succinate. The transaminase eluted from the DEAE-Sephadex column sometimes retains a slight contamination of heme protein which absorbs at 410 mμ. This can be removed by precipitating the enzyme with 85% ammonium sulfate, dissolving the precipitate in 0.02 M potassium phosphate buffer (pH 6.8), and dialyzing it against the same buffer. The solution can then be applied to a CM-Sephadex C-50 column (2 × 40 cm) equilibrated with the same buffer. The column was first washed with 600 ml of 0.02 M potassium phosphate (pH 6.8)–0.01 M KCl. The subforms of mitochondrial glutamate–aspartate transaminase were eluted using a linear gradient with 450 ml of 0.02 M potassium phosphate–0.01 M KCl in the mixing chamber and 450 ml of 0.02 M potassium phosphate–0.04 M KCl in the reservoir followed by another gradient with 0.02 M potassium phosphate–0.04 M KCl and 0.02 M potassium phosphate–0.06 M KCl. The orange impurity of the DEAE-Sephadex eluent remained at the top of the column. The protein profile of the elution pattern on CM-Sephadex is given in Figure 1. Transaminase activity traced the same pattern as that of the protein profile. The fractions were collected as indicated by the arrows in Figure 1, precipitated with 85% $(NH_4)_2SO_4$, dialyzed against water, and stored at −20° in 0.1 M $(NH_4)_2SO_4$. Under these conditions the enzyme remained stable for several months.

Electrophoretic Mobility. Horizontal starch gel electrophoresis at pH 8.6 was carried out according to the method described by Martinez-Carrion and Jenkins (1965). After electrophoresis the gels were cut horizontally and the upper layer was stained for enzymatic activity by the procedure of Decker and Rau (1963). The electrophoretic mobilities of the three fractions separated on CM-Sephadex and of supernatant glutamate–aspartate transaminase are seen in Figure 2. The first chromatographic fraction, which will be referred to as subform A, is the most cationic component and C, the last component eluted from CM-Sephadex, is the least positive at this pH. Fraction B migrates on starch gel intermediate to components A and C and contains a slight contamination of subform C. The separated proteins have the same cationic electrophoretic mobilities as in the unfractionated mixture and migrate opposite to the subforms of supernatant glutamate-aspartate transaminase.

Spectral Properties and Activity Measurements. Ab-

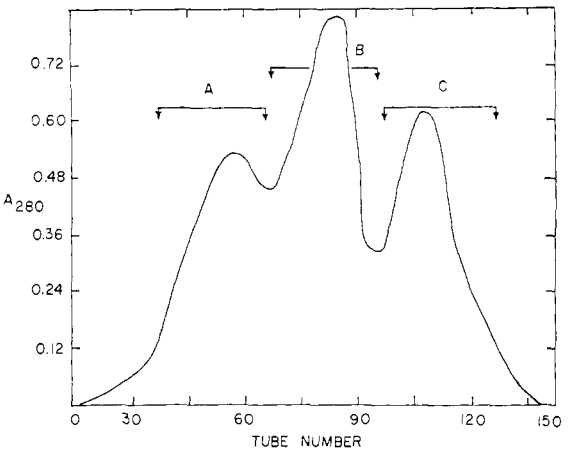

FIGURE 1: Elution profile of the three multiple forms of pig heart mitochondrial glutamate–aspartate transaminase on CM-Sephadex C-50. The arrows indicate the pooled fractions which are designated as A, B, and C in order of elution from the column. The conditions for separation are given in the text.

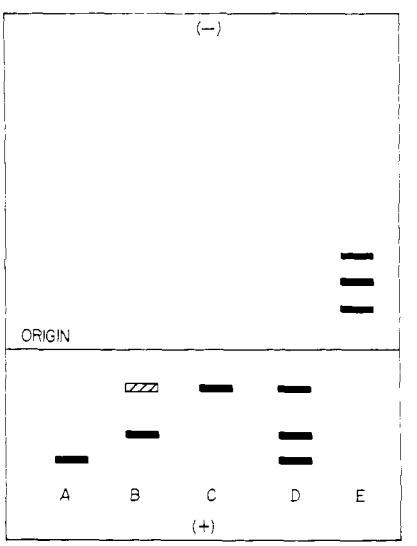

FIGURE 2: Starch gel electrophoresis pattern of purified pig heart glutamate–aspartate transaminase at pH 8.6. The gel was stained for protein with nigrosin. (A–C) Mitochondrial glutamate–aspartate transaminase fractions as eluted from CM-Sephadex. (D) Unfractionated mitochondrial glutamate–aspartate transaminase. (E) Supernatant pig heart aspartate aminotransferase.

TABLE I: Correlation of the Ratio of the Absorption Maxima at High and Low pH with the Specific Activity of the Enzyme.

Fraction	pH 5.0		pH 8.2		Sp. Act.[a]	
	$\frac{A_{280}}{A_{430}}$	$\frac{A_{430}}{A_{340}}$	$\frac{A_{280}}{A_{354}}$	$\frac{A_{280}}{A_{430}+A_{340}}$	pH 6.0	pH 7.5
A	11.8	1.92	9.0	7.75	97	100
B	11.3	2.18	8.4	7.80	116	120
C	10.1	2.74	7.65	7.73	138	140

[a] Assay consists of 3 ml of 0.05 M potassium phosphate buffer (pH 7.5 or pH 6.0), 0.0033 M aspartate, 0.0033 M ketoglutarate, NADH (8×10^{-5} M), and 5 μg of malate dehydrogenase. The specific activity is expressed as the number of moles of substrate used per minute per milligram of enzyme.

sorption spectra were measured in a Cary Model 15 recording spectrophotometer. As shown in Figure 3A, the mitochondrial glutamate–aspartate transaminase subforms exhibit similar absorptions at pH 5.0. All three fractions display maxima at 430 mμ characteristic of pyridoxal phosphate bound to the enzyme and another at 340 mμ. At pH 8.2 (Figure 3B), the absorption maximum of the enzyme shifts to 354 mμ whereas the maximum at 340 mμ remains unaltered. Subform A contains the greatest amount of 340-mμ-absorbing material whereas C has the least 340-mμ-absorbing material. Although the absorption maxima of the multiple forms are identical the ratios of the maxima designating the relative amounts of material absorbing at 340, 430, and 354 mμ differ (Table I). The absorption at 340 mμ has previously been identified in the supernatant glutamate–aspartate transaminase isozyme (Martinez-Carrion *et al.*, 1967) as pyridoxal phosphate bound to the enzyme in such a manner that it does not participate in catalysis. From now on this mode of binding will be referred to as "catalytically inactive" or "inactively bound" pyridoxal phosphate in contrast to the material which absorbs at 354 or 430 mμ which is "actively bound" or "catalytically active" pyridoxal phosphate. The ratio of the protein absorption to the sum of the maxima absorptions at low pH for all three fractions are identical (Table I). The specific activities of the three subforms at pH 6.0 and 7.5 can be correlated with the relative amount of material absorbing at 340 mμ. If the absorption at 340 mμ is due to "catalytically inactive" pyridoxal phosphate then subform A with the largest A_{280}/A_{430} and smallest A_{430}/A_{340} ratio should have the least specific activity, as reported in Table I.

Resolution of Holoaminotransferase and electrophoretic Mobility of the Apoenzyme. The transaminase was incubated with an excess of cysteinesulfinic acid for 15 min. The enzyme solution, made 0.5 M in potassium phosphate (pH 6.0) and saturated to 25% with $(NH_4)_2SO_4$, was left overnight at 5°. The enzyme was precipitated with 85% $(NH_4)_2SO_4$ and collected in a small amount of water. An excess of cysteine sulfinate was again added and incubated with the enzyme. The pH of the solution was then adjusted to 5.5 with an equal amount of 1 M potassium phosphate and the ionic

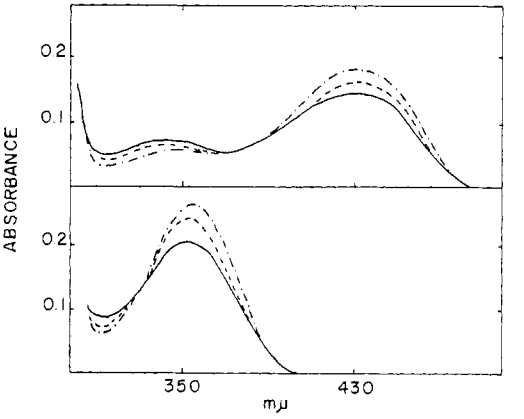

FIGURE 3: Spectral studies. top: Absorption spectra of mitochondrial glutamate–aspartate transaminase subforms at pH 5.0 in 0.1 M sodium acetate buffer. (——) Chromatographic fraction A; (– – –) fraction B; (·–·–·) fraction C. bottom: Subfractions A (——), B (– – –), and C (·–·–·) at pH 8.2 in 0.1 M Tris buffer. Spectra were measured at concentrations of 1.3 (A), 1.41 (B), and 1.39 mg/ml (C).

strength was increased by adding 40% $(NH_4)_2SO_4$. After incubation for 10 hr in the cold the enzyme was precipitated as indicated before, dissolved in 0.1 M potassium phosphate (pH 6.8) and dialyzed against the same buffer for 15 hr. This treatment resulted in 92–99% resolution. After resolution of the three subforms of mitochondrial glutamate–aspartate transaminase, none of the apoenzymes displayed any absorption at 430 or 354 mμ but the absorption at 340 mμ remained. Therefore, only "actively bound" pyridoxal phosphate could be removed by the above procedure. The migration in starch gel electrophoresis of the apoenzymes of the three subforms resembled that of the parent holoenzymes. This indicates that at least at this pH the change in net charge upon removal of the coenzyme is negligible.

Rate of Recombination of Pyridoxal Phosphate with Apoenzyme. The following studies were undertaken to determine if the variation in "actively" and "inactively" bound pyridoxal phosphate might affect the rate at which the apoenzymes recombine with the coenzyme. The procedure followed was similar to that of Martinez-Carrion *et al.* (1967). The apoenzyme ($2–3 \times 10^{-8}$ M)

TABLE II: Bimolecular Rate Constants for Recombination of Apoenzyme with Pyridoxal Phosphate in Tris Buffer, pH 7.5.[a]

Fraction	I	II	III	Average[b]
(A) Mitochondrial glutamate–aspartate transaminase	1.05×10^5	9.9×10^4		1.02×10^5
(B) Mitochondrial glutamate–aspartate transaminase	5.1×10^4	6.1×10^4	6.62×10^4	5.6×10^4
(C) Mitochondrial glutamate–aspartate transaminase	5.15×10^4	7.45×10^4		6.3×10^4

[a] The results in columns I, II, and III are calculated from three different experiments using the same apoenzyme preparation. [b] The values of the bimolecular rate constants are expressed in M^{-1} sec^{-1}.

TABLE III: Amino Acid Composition of Multiple Forms of Mitochondrial Glutamate–Aspartate Transaminase.

	(A) Mitochondrial glutamate–aspartate tranaminase		(B) Mitochondrial glutamate–aspartate transaminase		(C) Mitochondrial glutamate–aspartate transaminase	
Amino Acid	Calcd Residues/50,000 mol wt	Nearest Integer	Calcd Residues/50,000 mol wt	Nearest Integer	Calcd Residue/50,000 mol wt	Nearest Integer
Arginine	19.8	20	19.7	20	20.5	21
Histidine	10.7	11	10.7	11	11.2	11
Lysine	29.2	29	29.1	29	29.3	29
Phenylalanine	20.4	20	21.0	21	19.8	20
Tyrosine	12.3	12	12.4	12	12.4	12
Leucine	30.4	30	29.6	30	30.1	30
Isoleucine	24.0	24	24.1	24	24.0	24
Methionine	11.4	11	11.6	12	11.8	12
Cysteine[a]	4.7	5	5.2	5		
Valine	26.9	27	27.0	27	27.8	28
Alanine	35.0	35	35.0	35	36.4	36
Glycine	35.2	35	34.2	34	34.9	35
Proline	19.4	19	17.5	18	18.4	18
Glutamate	42.4	42	42.0	42	41.5	42
Serine[b]	23.0	23	23.4	23	23.4	23
Threonine[b]	16.1	16	17.3	17	17.4	17
Aspartate	38.7	39	40.1	40	38.6	39

[a] Calculated according to the method of Boyer. [b] Extrapolated to zero hydrolysis. (All values are the average of 24-, 48-, and 72-hr hydrolyses in 6 N HCl except where indicated.)

was incubated in 0.05 M Tris-HCl (pH 7.5) at 5° with pyridoxal phosphate at concentrations of 5×10^{-7} to 1×10^{-6} M. At various time intervals 2.5 ml of assay mixture containing 0.1 M phosphate buffer (pH 7.5), equilibrated at 30°, was added directly to 0.5-ml samples. The samples were immediately assayed in a Cary 15 spectrophotometer thermostated at 30°. The assay, containing 0.1 M phosphate buffer, inhibits further recombination of coenzyme with apoenzyme. With an excess of pyridoxal phosphate, the recombination follows a pseudo-first-order reaction. A plot of the logarithm of the apoenzyme at time t against time is linear with a slope equal to the first-order rate constant. Division of the pseudo-first-order rate constant by the concentration of pyridoxal phosphate gives the value for the bimolecular rate constant. The bimolecular rate constants (Table II) are similar for apo B and C but doubled for apoenzyme A.

Amino Acid Analysis. Samples of protein in 6 N HCl were hydrolyzed for 24, 48, and 72 hr at 110° in tubes sealed under vacuum. The amino acid compositions of the hydrolysates were determined in a Technicon Autoanalyzer. Sulfhydryl groups were estimated with *p*-mercuribenzoate according to the method of Boyer (1954). Values of serine and threonine were obtained by extrapolation of their recoveries to zero time due to partial destruction of these amino acids during acid hydrolysis. The number of leucine and isoleucine residues were evaluated only on 72-hr hydrolysis time because these peptide bonds are somewhat resistant to hydrolysis. Methionine peak areas were taken as the sum of the peak areas for methionine and methionine sulfoxide. Table

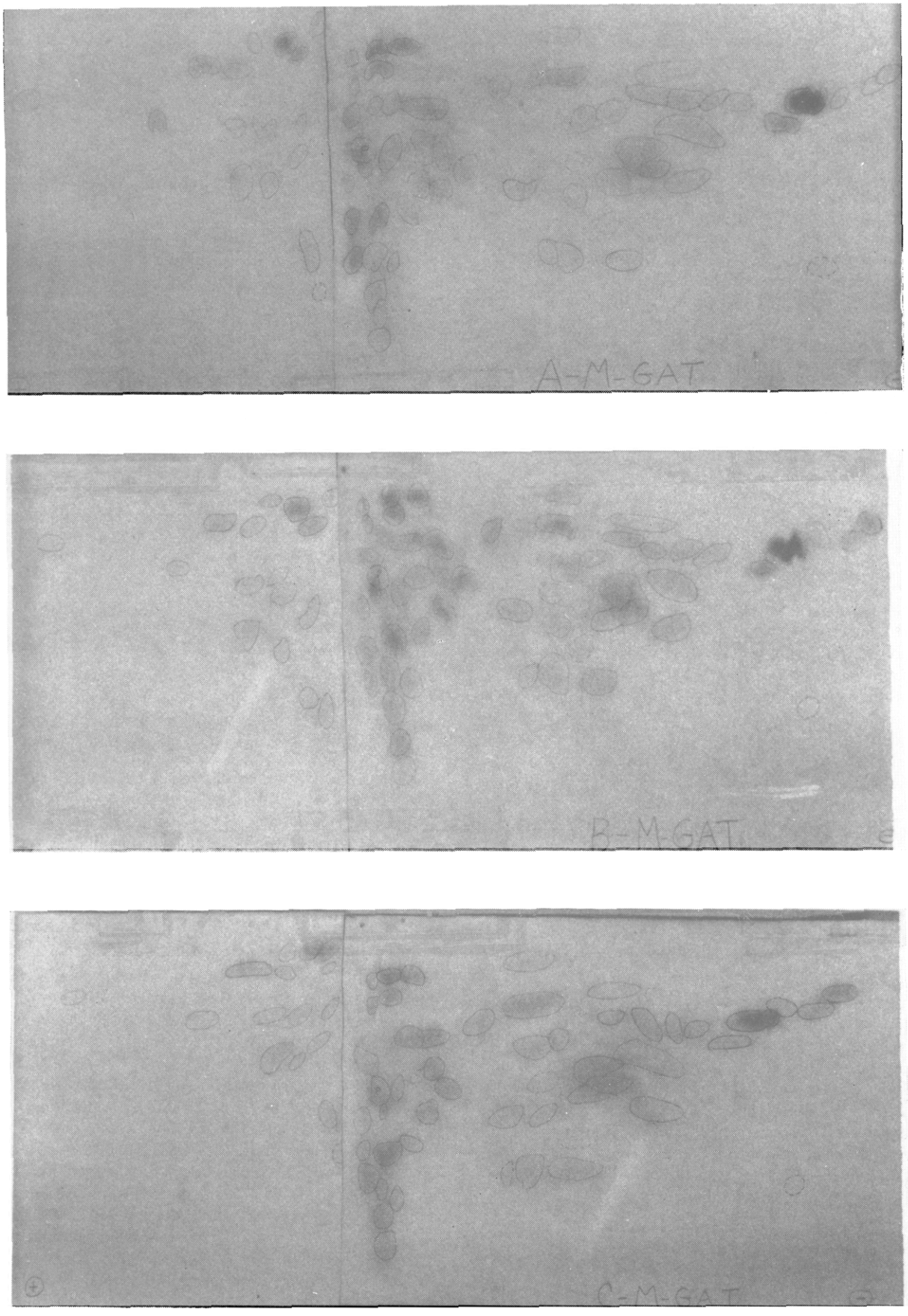

FIGURE 4: Tryptic peptide maps of subforms of mitochondrial glutamate–aspartate transaminase. Peptide designated as P indicates the peptide containing pyridoxal phosphate.

III shows the amino acid composition of the three subforms of mitochondrial glutamate–aspartate transaminase based on a molecular weight of 50,000. Within the limits of experimental error, no significant difference can be noted in the amino acid content of the multiple forms.

Peptide Maps. The procedure for peptide mapping of tryptic digests of fractions A–C was similar to that of Martinez-Carrion and Tiemeier (1967) except where indicated. Samples (8 mg) of the mitochondrial glutamate–aspartate transaminase subforms were dialyzed against 0.05 M potassium phosphate buffer (pH 6.8) and 0.05 M sodium borohydride for 20 min. The reduced samples were then dialyzed against 0.05 M potassium phosphate (pH 6.8) to remove the excess sodium borohydride. The enzyme was heat denatured in boiling water for 3 min. After digestion for 30 hr with L-(1-tosylamido-2-phenyl) ethyl chloromethyl ketone treated trypsin, the hydrolytic enzyme was precipitated with formic acid and the residual unreacted enzyme and denatured trypsin were removed by centrifugation. Electrophoresis was carried out in a Savant Instruments, Inc., high-voltage paper electrophoresis apparatus using a potential of 35 V/cm for 1 hr. After descending chromatography, the maps were dried and the fluorescing pyridoxal peptide was located with a short-wave ultraviolet lamp. The pyridoxal peptide, marked as P in Figure 4, has the same position in all three maps. Although pyridoxal phosphate which is "catalytically active" and "catalytically inactive" can be distinguished by their different absorption maxima only one peptide could be located which contained pyridoxal phosphate. A possible explanation of this could be that either the mode of binding of pyridoxal phosphate to the peptide does not affect its migration or that pyridoxal phosphate which is "catalytically inactive" does not reduce with $NaBH_4$ and being labile is removed during the digestion procedures. For all three subforms the total number of major peptides (46) represented by the solid tracings, and minor peptides (21) corresponding to the dotted tracings in Figure 5, are equal.

Optical Rotatory Dispersion Studies. Studies on the optical rotatory dispersion properties of the multiple forms of mitochondrial glutamate–aspartate transaminase were measured on a Cary Model 60 recording spectropolarimeter. A group of Cotton effects were recorded in the spectral region from 550 to 200 mμ for all three mitochondrial glutamate–aspartate transaminase subforms. No changes in optical rotatory dispersion pattern were noted around the 232-mμ range. A positive Cotton effect, centered around 430 mμ at low pH and 350 mμ at high pH, was found for the three mitochondrial glutamate–aspartate transaminase subforms. Removal of the coenzyme from holoaminotransferase did not alter the rotatory dispersion in the ultraviolet. Rotations were converted into values of the reduced mean residue rotation, $[m']$, by the equation

$$[m'] = \left(\frac{3}{n^2 + 2}\right)\frac{MRW}{100}(\alpha)$$

where $[\alpha]$ is the specific rotation at a designated wavelength, MRW is the mean residue molecular weight (calculated from the amino acid composition as 113.5), and n is the refractive index of the solvent at the same wavelength. The A–C subforms of mitochondrial glutamate–aspartate transaminase displayed optical rotatory dispersion bands at 232 mμ with mean residue rotations of about −5500° (Table IV) and their crossover point is at 223 mμ (Figure 5).

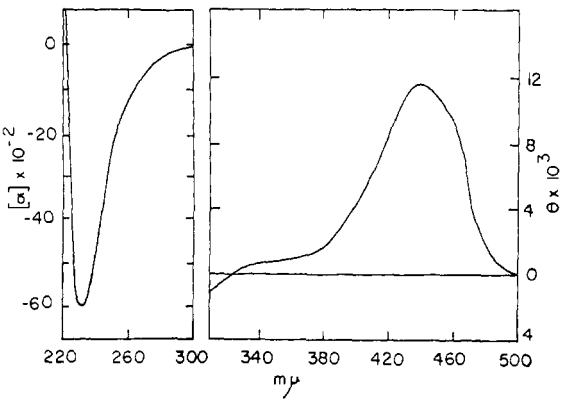

FIGURE 5: Optical rotatory dispersion of pig heart mitochondrial glutamate–asparatate transaminase in the low-wavelength region and circular dichroism pattern in the 300–500-mμ region at pH 5.0 in 0.1 M sodium acetate buffer, 30°. All three subforms exhibit identical dispersion and dichroic patterns to that reported above with respect to maxima. The protein concentration range was 0.9–1.1 mg/ml using 1- and 10-mm silica cells.

TABLE IV: Optical Activities of Glutamate–Aspartate Transaminase Subforms.[a]

Glutamate–Aspartate Transaminase	$[m']_{232}$ mμ	$(\Delta\epsilon/\epsilon)_{430}$ mμ	$[\theta]_{430}$ mμ[b]
Mitochondrial A	−5400	3.27×10^{-3}	133
Mitochondrial B	−5500	3.20×10^{-3}	150
Mitochondrial C	−5430	3.23×10^{-3}	160
Supernatant [α]	−5500	3.30×10^{-3}	165

[a] The optical rotatory dispersion parameter at 232 mμ remains insensitive to pH changes. All measurements were made at 30°. [b] The ellipticity at 430 mμ is measured at pH 5.0 in 0.1 sodium acetate buffer.

Circular Dichroism Measurements. Studies of the optical activity of the A–C forms of mitochondrial glutamate–aspartate transaminase by circular dichroism methods were performed on a Cary Model 60 spectropolarimeter with a special circular dichroism attachment. The circular dichroism pattern of mitochondrial glutamate–aspartate transaminase from 550 to 320 mμ at pH 5.0 and 9.1 is similar to that of the supernatant transaminase (Ivanov *et al.*, 1967) with a positive ellipticity band at 430 mμ at pH 5.0 (Figure 5) that shifts to 355 mμ at a more basic pH mimicking the absorption spectral properties. Although the three subforms exhibit ellipticity bands indicative of the binding of pyri

TABLE V: Reaction of Mitochondrial Glutamate–Aspartate Transaminase Subforms with Amino Acid Pseudosubstrates.[a]

Subform	Amino Acid	Amino Acid Concn (M)	First-Order Rate Constant (min^{-1})	$t_{1/2}$ (min)
A	L-Methionine	0.033	5.93×10^{-1}	1.17
B	L-Methionine	0.033	6.15×10^{-1}	1.12
C	L-Methionine	0.033	6.7×10^{-1}	1.04
A	L-Alanine	0.10	3.76×10^{-1}	1.84
B	L-Alanine	0.10	3.5×10^{-1}	1.97
C	L-Alanine	0.10	3.52×10^{-1}	1.92
A	L-Serine	0.091	5.25×10^{-2}	13.4
B	L-Serine	0.091	5.92×10^{-2}	11.6
C	L-Serine	0.091	5.1×10^{-2}	13.6

[a] Followed in a Cary Model 15 spectrophotometer at 355 mμ in 0.1 M Tris buffer, pH 8.2, at 30°.

doxal phosphate to the active site in an asymmetric environment, the mean residue molecular ellipticity in degrees, $[\theta]$, differs. The measured ellipticity, $\theta°$, is related to the molecular ellipticity by the expression

$$[\theta] = \frac{\theta° M}{10 l C'}$$

where M is the mean residue molecular weight of the sample, l is the path length in the sample solution in cm, and C' is the concentration in g/cm³. $[\theta]$ has the dimensions of (deg cm²)/dmole of amino acid residue. The relation between the ellipticity and the differential dichroic absorption can be expressed (Velluz *et al.*, 1965) as

$$[\theta] = 2.303\frac{4500}{\pi}(\epsilon_l - \epsilon_r)$$

where $\epsilon_l - \epsilon_r$ is the difference in extinction coefficient for the absorption of left and right circularly polarized light. The circular dichroism and optical rotatory dispersion parameters for the mitochondrial glutamate–aspartate transaminase subforms are listed in Table IV. Rotations at 232 mμ are analogous for the three mitochondrial glutamate–aspartate transaminase subforms and can be interpreted in terms of the three enzymes possessing similar over-all tertiary and secondary structure. Variation in the molecular ellipticity at 430 mμ corresponds to the amounts of "actively" and "inactively" bound pyridoxal phosphate among the subforms.

Quaternary Structure and Molecular Weight of the Multiple Forms. The molecular weights of the three subforms of glutamate–aspartate transaminase were determined by using sucrose gradients by the technique of Martin and Ames (1961). The transaminase peak was located both by reading protein absorption at 280 mμ and by assaying for activity. In the determination of the distance of migration the peak activity or absorbance was used as the center of the moving band. Under the conditions used, all three forms of mitochondrial glutamate–aspartate transaminase were found to have a molecular weight of 100,000 ± 10,000. In addition, the molecular weight of fraction C was determined by gel filtration on Sephadex G-200. The column size was 2.5 × 50 cm and correlation was made between elution volume and molecular weight according to the method of Andrews (1965). An estimate of a molecular weight of 110,000 ± 11,000 for subform C was calculated using gel filtration techniques which is in agreement with the value obtained by sucrose density gradient measurements.

Immunodiffusion Studies. Antisera to subforms B and C were prepared by intramuscular injection in separate rabbits of 8–9 mg of each antigen in Freund's complete adjuvant (Ouchterlony, 1949). In agar diffusion plates a single fused precipitin line formed between the subforms of mitochondrial glutamate–aspartate transaminase whereas supernatant glutamate–aspartate transaminase did not cross-react with mitochondrial enzyme antiserum.

Reactions with Other Amino Acids. Glutamate or aspartate are not suitable substrates for following the direct reaction of the amino acid with the prosthetic group of the enzyme by ordinary spectrophotometric methods because they react instantaneously. In the presence of certain amino acids which are not the normal substrates of mitochondrial glutamate–aspartate transaminase, the half-reaction between the pyridoxal form of the enzyme with the amino acid can be followed spectrophotometrically as a function of time. Since the pyridoxal enzyme absorbs at 354 mμ at pH 8.2 and the pyridoxamine form of the enyzme absorbs at 330 mμ the decrease in absorbance at 354 mμ is related to the rate of the forward reaction. If conditions are chosen such that the amino acid is in great excess of the pyridoxal enzyme the back reaction will be negligible and the reaction is observed to be first order in pyridoxal enzyme. If the logarithm of the difference between the absorbance at time, t, minus the absorbance at infinite

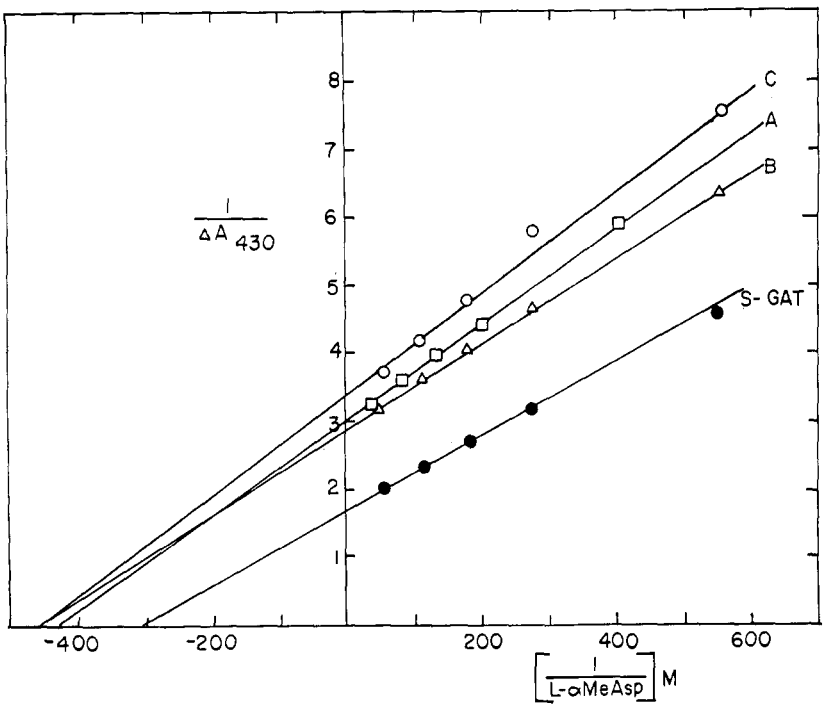

FIGURE 6: Computation of the dissociation constants for aspartate aminotransferase–L-α-methylaspartate complex in 0.4 M borate buffer, pH 9.1, at 30°. (□—□) (A) mitochrondrial glutamate–aspartate transaminase, (△—△) (B) mitochondrial glutamate aspartate transminase, (○—○) (C) mitochondrial glutamate–aspartate transaminase, and (●—●) supernatant glutamate–aspartate transaminase. Protein concentrations used were about 0.2 mg/ml.

time, which is proportional to the amount of pyridoxal enzyme present, is plotted against the time of reaction a straight line is obtained with a slope equal to the first-order rate constant. To the pyridoxal enzyme (2–3 mg) the amino acid was added and the reaction was followed spectrophotometrically. The first-order rate constants and half-lives for the reaction of aminotransferase subforms A, B, and C with L-methionine, L-serine, and L-alanine are equivalent (Table V).

Interaction with α-Methylaspartate. A kinetic analysis of mitochondrial aspartate aminotransferase by steady-state methods may be subject to error because reciprocal plots of the variation of the initial velocity as a function of the concentration of keto acid holding the amino acid constant yields a family of lines which converge (Wada and Morino, 1964). This apparent behavior seems to be in contrast to the "Ping-Pong bi-bi" type mechanism displayed by the supernatant enzyme but could be explained in terms of inhibition by oxaloacetate rather than ternary complex formation. In our laboratory a spectrophotometric analysis of the kinetic behavior of the mitochondrial glutamate–aspartate transaminase was preferred. This method has the advantages of directly measuring the complex formed between enzyme and substrate so that the true dissociation constant of the reaction can be calculated rather than the Michaelis constant. Mtiochondrial glutamate–aspartate transaminase, in the presence of α-methylaspartate, forms a complex which absorbs at 360 mμ and 430 mμ as has been found with supernatant glutamate–aspartate transaminase (Jenkins *et al.*, 1959; Fasella *et al.*, 1966; Ivanov *et al.*, 1967). Since α-methylaspartate can form some of the enzyme–substrate inter mediates with mitochondrial glutamate–aspartate transaminase involved in transamination, but not to or beyond the removal of the α-hydrogen from the substrate, the absorptions at 360 and 430 mμ represent an equilibrium mixture of at least two enzyme–substrate intermediates previous to the tautomerization step in the reaction mechanism. By titrating the pyridoxal form of the enzyme at pH 9.1 with α-methylaspartate the absorbance at 430 mμ increases whereas the absorbance at 360 mμ decreases. The dissociation constant and extinction coefficient of the complexes can be determined by the following analysis. The pyridoxal form of the enzyme, E, interacts with α-methylaspartate, A, to yield the complex EA where

$$E + A \rightleftharpoons EA$$

The concentration of the complex EA at any time t is related to the change in absorbance before and after addition of α-methylaspartate, ΔA, and the difference in extinction coefficients between the free enzyme (e_2) and [EA](e_1) by

$$[EA] = \Delta A/(e_1 - e_2) \quad (1)$$

If the total enzyme concentration is equal to the sum of the enzyme bound to the amino acid, [EA], and free enzyme, [E], at any time t then

$$[E_t] = [EA] + [E] \quad (2)$$

Substituting K_{diss_n}[E][A]/[EA] and eq 1 into 2 and rearrangement gives

$$[E_t]/\Delta A = \frac{K_{diss_n}/\alpha\text{-methylaspartate} + 1}{e_1 - e_2} \quad (3)$$

By plotting $1/\Delta A$ *vs.* $1/\alpha$-methylaspartate (Figure 6) the K_{diss_n} can be determined from the intersection on the abscissa axis when $y = 0$. Each subform of mitochondrial glutamate–aspartate transaminase was titrated with DL-α-methylaspartate in 0.1 M borate buffer (pH 9.1). After each addition of amino acid, the spectrum was recorded between 550 and 350 mμ. Enzyme concentrations were calculated on the basis of their molar extinction coefficient of bound pyridoxal phosphate at pH 5.0. Figure 6 compares the dissociation constants and extinction coefficients of the multiple forms enzyme–α-methyl aspartate complexes and it can be seen that they are identical but different from those obtained with supernatant glutamate–aspartate transaminase under the same experimental conditions.

Discussion

None of the possible explanations that have been given for the differences between isozymes or multiple forms can be used to completely interpret mitochondrial glutamate–aspartate transaminase subform variances. All three subforms are present in the initial tissue extract and, as far as can be detected using a histochemical staining technique, the relative proportions of the fractions do not change throughout the purification steps. (Under our conditions three cationic bands can be detected only when starch gel electrophoresis is allowed to proceed for at least 3 hr. Otherwise, subforms A and B will appear as one activity band.) The subforms of mitochondrial glutamate–aspartate transaminase do not seem to be artifacts of starch gel electrophoresis because they can be separated by ion-exchange chromatography, differ in absorption properties and vary in specific activity. The binding of pyridoxal phosphate to the apoenzymes distinguishes the three mitochondrial glutamate–aspartate transaminase chromatographic fractions. There appears to be two types of binding of pyridoxal phosphate to the enzyme, the amount of each varying among the subforms. One type can probably be ascribed to a phosphopyridoxal–aldimine enzyme linkage (Martinez-Carrion and Tiemeier, 1967) and is sensitive to changes in pH exhibiting maxima at 354 (pH 8.2) and 430 mμ (pH 5). The other type of linkage, termed "inactively bound" pyridoxal phosphate manifests its peak absorption at 340 mμ which is insensitive to pH alterations. Although the mitochondrial glutamate–aspartate transaminase subforms differ in specific activities they possess identical amounts of bound pyridoxal phosphate because: (1) incubation of the subforms with pyridoxal phosphate previous to assaying for transaminase activity does not alter the specific activity of any of the subforms; (2) if pyridoxal phosphate is removed from the holoenzymes and then recombined with the apoenzyme, the new holoenzyme of each subform retains the specific activity it had before the removal of the coenzyme; and (3) the total pyridoxal phosphate content, which is the sum of the actively and inactively bound pyridoxal phosphate per mg of protein, is identical.

During the separation procedure we observed only one electrophoretic and chromatographic band for each mitochondrial glutamate–aspartate transaminase subform. At no time was there indication that any one band could be further separated into two fractions, one containing totally active and the other totally inactive enzyme. In other words, an entirely active enzyme with no 340-mμ absorbance or a completely inactive enzyme with only 340 mμ could not be isolated. This could mean that although subforms A–C are made up of populations of molecules containing various proportions of actively and inactively bound pyridoxal phosphate, each subform is structurally homogeneous.

Since the various subforms maintained their electrophoretic individuality after the "active" coenzyme was removed, it appears that the apoenzymes of the various subforms still differ and excludes the possibility that the mitochondrial glutamate–aspartate transaminase subforms may be produced only by the different modes of binding of pyridoxal phosphate to the same protein.

The dissociation constant for α-methylaspartate and the first-order rate constants for L-alanine, L-methionine, and L-serine are equal for the three mitochondrial glutamate–aspartate transaminase subforms because these quantities, as measured by the direct spectrophotometric method, reflect only the amount of active enzyme present. On the other hand, their specific activities are calculated on the basis of total enzyme content, consisting of both the active and inactive enzyme, and, therefore, differ.

The dissociation constants for α-methylaspartate for the mitochondrial and supernatant enzymes are 2.2×10^{-3} M for the former and 3.03×10^{-3} M for the latter under the same experimental conditions. The K_m's for the natural substrates calculated by kinetic data for the glutamate–aspartate transaminase isozymes are also different (Nisselbaum and Bodansky, 1966; Boyde, 1968).

Dissimilarities among the three mitochondrial glutamate–aspartate transaminase fractions as to the amount of "actively bound" pyridoxal phosphate can account for diversities in $[\theta]_{430}$ (Table IV). The circular dichroism spectra does not display any ellipticity centering around 340 mμ. The γ form of supernatant glutamate–aspartate transaminase contains large amounts of 340-mμ-absorbing material with a $[\theta]_{340}$ mμ value of 14.5.[1] Ivanov *et al.* (1967) report this inert form, absorbing at 340 mμ, as devoid of optical activity. Since $[\theta]$ at 340 mμ is so small, it is possible that for mitochondrial glutamate–aspartate transaminase it would appear as transparent rather than optically active because the relative amount of 340-mμ absorbance for this isozyme is much less than for γ supernatant glutamate–aspartate transaminase. The circular dichroism pattern at 430 mμ

[1] M. Martinez-Carrion, D. Tiemeier, and D. Peterson, 1969, unpublished data.

(pH 5.0) is well in agreement with absorption spectral data. The mean residue rotation at 232 mμ and crossover point from negative to positive rotation at 223 mμ using optical rotatory dispersion techniques is calculated to the same values for the mitochondrial glutamate–aspartate transaminase fractions and imply that these rotations are mainly due to contribution by the α helix as in the supernatant isoenzyme. Optical rotatory dispersion studies have pointed out that at least by these criteria: (1) no detectable conformational differences can be noted among the glutamate–aspartate transaminase subforms; (2) the subforms do not change conformation with changes in pH; and (3) the addition of pyridoxal phosphate to the transaminase apoenzymes does not seem to affect the conformation of the protein as can also be seen for supernatant glutamate–aspartate transaminase (Martinez-Carrion, 1968). In conclusion, therefore, optical rotatory dispersion data have indicated that the mitochondrial glutamate–aspartate transaminase subforms are not "conformers" but are structurally alike. An emphasis should be made here that erroneous results can be obtained in making physical measurements with enzyme preparations which are old. With an old preparation of subform A, a $[mi']_{232}$ as low as −4200° was obtained whereas with a fresh preparation of enzyme the mean residue rotation was identical with that of the other mitochondrial glutamate–aspartate transaminase enzyme fractions.

In comparing the two transaminase isozymes, it is remarkable that two proteins of such diverse amino acid composition and sequence (Martinez-Carrion and Tiemeier, 1967) would be so similar in the binding of pyridoxal phosphate and, as indicated by the optical rotatory dispersion measurement in the ultraviolet region, in over-all conformation (Table IV).

The chemical composition of the three mitochondrial glutamate–aspartate transaminase components, as revealed by the amino acid composition, are similar and in agreement with that computed for the unfractionated enzyme by Martinez-Carrion and Tiemeier (1967). Peptide maps of tryptic digests of each fraction did not show differences in the number or location of the peptides. This evidence rules out that the variances in electrophoretic mobility for each mitochondrial glutamate–aspartate transaminase subform is due to a different glutamine and/or asparagine content. Double diffusion on agar gel of A–C subforms against antisera to subform B reveal precipitin lines with complete fusion indicative of similar immunochemical specificity among the enzymes.

Sucrose gradient studies strengthens the concept that all subforms of mitochondrial glutamate–aspartate transaminase are dimers of similar or identical subunits with a molecular weight of 100,000.

Pig heart supernatant glutamate–aspartate transaminase has been compared to the unfractionated mitochondrial enzyme by Martinez and Tiemeier (1967) and found to differ significantly in terms of structure. Although both isozymes have many physical and chemical dissimilarities both systems possess multiple forms. The supernatant transaminase consists of at least four electrophoretically distinguishable fractions which, have been isolated and characterized (Martinez-Carrion *et al.*, 1967). Comparison of the structural and functional similarities within the multiple form series of the supernatant and mitochondrial isozymes point out remarkable parallelisms. The subforms of supernatant glutamate–aspartate transaminase have been found to differ only in specific activity, the amount of "inactively bound" pyridoxal phosphate to the enzyme, behavior in 8 M urea and kinetics of recombination with the coenzyme. The supernatant glutamate–aspartate transaminase subforms vary in the amount of 340-mμ-absorbing material, the γ subform of supernatant glutamate–aspartate transaminase being comparable to the A fraction of mitochondrial glutamate–aspartate transaminase. The α subform of supernatant glutamate–aspartate transaminase has the highest specific activity and closely resembles the C subform of mitochondrial glutamate–aspartate transaminase.

The entire chemical and physicochemical evidence does not point to major conformational or structural differences that could explain the finite electrophoretic and chromatographic individuality of the multiple forms of both supernatant glutamate–aspartate transaminase and mitochondrial glutamate–aspartate transaminase. On the other hand, this diversity can be due to subtle conformational or covalent structural variations in localized regions of the enyzme, like around the active site, that remained undetected by our methods. A further kinetic examination of the behavior of the subform systems might prove to be most beneficial in resolving the functional significance of the multiple forms *in vivo*.

References

Andrews, P. (1965), *Biochem. J. 96*, 595.

Boyd, J. W. (1966), *Biochim. Biophys. Acta 113*, 302.

Boyde, T. R. C. (1968), *Biochem. J. 106*, 581.

Boyer, P. D. (1954), *J. Amer. Chem. Soc. 76*, 4331.

Cohn, R. D., Kaplan, N. O., Levine, L., and Zwilling, L. (1962), *Science 136*, 962.

Dawson, D. M., Eppenberger, H. M., and Kaplan, N. O. (1967) *J. Biol. Chem. 242*, 210.

Decker, L. E., and Rau, E. M. (1963), *Proc. Soc. Exptl. Biol. Med. 112*, 144.

Fasella, P., Giartosio, A., and Hammes, G. (1966), *Biochemistry 5*, 197.

Flatmark, T. (1967), *J. Biol. Chem. 242*, 2454.

Fleischer, G. A., Potter, C. S., Wakim, K. G., Pankow, M., and Osborne, D. (1960), *Proc. Soc. Exptl. Biol. Med. 103*, 229.

Ivanov, V. I., Breusov, Y., Karpeiskii, M., Polyanovskii, O. L. (1967), *Molekul. Biol. 1*, 588.

Jacobson, K. B. (1968), *Science 159*, 324.

Jenkins, W. T., Yphantis, D. A., and Sizer, I. W. (1959), *J. Biol. Chem. 234*, 51.

Kitto, G. B., Wassarman, P. M., and Kaplan, N. O. (1966), *Proc. Natl. Acad. Sci. U. S. 56*, 578.

Margoliash, E., and Lustgarten, J. (1961), *Ann. N. Y. Acad. Sci. 94*, 731.

Martin, P. G., and Ames, B. N. (1961), *J. Biol. Chem. 236*, 1372.

Martinez-Carrion, M. (1968), *in* Symposium on Pyridoxal Enzymes, Yamada, K., Katunuma, N., and Wada, H., Ed., Tokyo, Maruzen, p 19.

Martinez-Carrion, M., and Jenkins, W. T. (1965), *J. Biol. Chem. 240*, 3528.

Martinez-Carrion, M., Riva, F., Turano, C., and Fasella P. (1965), *Biochem. Biophys. Res. Commun. 20*, 206.

Martinez-Carrion, M., and Tiemeier, D. (1967), *Biochemistry 6*, 1715.

Martinez-Carrion, M., Turano, C., Chiancone, E., Bossa, F., Giartosio, A., Riva, F., and Fasella, P. (1967), *J. Biol. Chem. 242*, 2397.

Nisselbaum, J. S., and Bodansky, O. (1964), *J. Biol. Chem. 239*, 4232.

Nisselbaum, J. S., and Bodansky, O. (1966), *J. Biol. Chem. 241*, 2661.

Ouchterlony, O. (1949), *Acta Pathol. Microbiol. Scand. 26*, 507.

Penhoet, E., Kochman, M., Valentine, T., and Rutter, W. J. (1967), *Biochemistry 6*, 2940.

Velluz, L., Legrand, M., and Grossjean, M. (1965), Optical Circular Dichroism, New York, N. Y., Academic.

Wada, H., and Morino, Y. (1964), *Vitamins Hormones, 22*, 411.

Subunits of Rat Liver Mitochondrial Malate Dehydrogenase*

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ABSTRACT: Considerable evidence has suggested that mammalian mitochondrial malate dehydrogenase is composed of subunits. In the present investigation the molecular weight of highly purified rat liver malate dehydrogenase has been studied in several systems by sedimentation equilibrium and by sedimentation diffusion. The intact molecule has a molecular weight of 66,300. The molecule is dissociated at pH 2, or in 5 or 7 M guanidine hydrochloride or 8 M urea to half-molecules. The half-molecules present at pH 2 or in 8 M urea are inactive.

The inactivated enzyme can be reactivated by neutralization in the one case or by removal of the denaturing reagent in the other. The data presented indicate that the extent of reactivation which occurs on neutralization of enzyme solutions exposed to a pH 2 environment is dependent upon the length of time at pH 2. In a pair of sedimentation equilibrium experiments the molecular weight of beef heart malate dehydrogenase was shown to be 64,000 in 0.1 M sodium citrate–0.002 M 2-mercaptoethanol (pH 6.25). In 5 M guanidine hydrochloride a value of 35,700 was obtained.

Purified preparations of rat liver mitochondrial malate dehydrogenase can be separated into five bands of catalytic activity when subjected to starch gel electrophoresis. Of these five isozymes, three are responsible for about 90% of the total catalytic activity. Such malate dehydrogenase isozymes have previously been reported for a number of species (Grimm and Doherty, 1961; Thorne *et al.*, 1963).

A great deal of evidence has accumulated which indicates that malate dehydrogenase is composed of subunits. However, some confusion has arisen over the number of subunits in the molecule.

Munkres and Richards (1965) have reported that the malate dehydrogenase of *Neurospora crassa* is composed of four subunits. Further, Munkres (1965) has shown that the enzyme is composed of two types of polypeptide chains (designated α and β) and has the over-all structure $\alpha_3\beta$. Recently Siegel (1967) has reported that beef heart malate dehydrogenase is composed of four subunits, the molecule being dissociated in 4 M urea.

Dévényi *et al.* (1966) have proposed that the pig heart enzyme is composed of two similar or identical subunits on the basis of tryptic fingerprint studies. Thorne *et al.* (1963) have studied the binding of NADH to the pig heart enzyme, and have determined that there are two binding sites per molecule.

The present paper deals with dissociation studies of rat liver malate dehydrogenase. These studies, conducted in 4 and 8 M urea, in 5 and 7 M guanidine hydrochloride, and in a series of experiments at pH 2.0 indicate that the rat liver malate dehydrogenase molecule is composed of two subunits of equal molecular weight.

Experimental Procedures

Materials

Rat liver malate dehydrogenase was purified by the

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