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Mitochondrial Glutamate–Aspartate Transaminase. I. Structural Comparison with the Supernatant Isozyme*

M. Martinez-Carrion and D. Tiemeier

ABSTRACT: Mitochondrial pig heart glutamate–aspartate transaminase (L-aspartate:2-oxoglutaratea minotransferase, EC 2.6.1.1) can be isolated in a state of purity suitable for detailed structural work. The absorption spectrum of this transaminase at pH 5 is identical with that of its supernatant isozyme but at pH 8.2 it is slightly different. This small discrepancy in spectra can be best explained in terms of some pyridoxal phosphate binding by the mitochondrial isozyme at sites other than the active center. The A_{280}:A_{355} ratio is 8.7 at pH 8.2. The molecular absorbancy coefficient due to the bound pyridoxal phosphate is 8350 ± 700 at 355 mμ (at pH 5) and 8050 ± 300 at 435 mμ (at pH 8.2). Pyridoxal phosphate is probably bound in an azomethine link to the protein and this bond can be reduced with sodium borohydride. This reduction shifts the absorption maxima to 330 mμ and renders the enzyme inactive. The purified enzyme yields two cationic protein bands that possess transaminase activity in starch gel electrophoresis at pH 8.6. The N-terminal amino acid is serine and the amino acid composition of this enzyme is distinct from that of the supernatant isozyme. Tryptic digests of both isozymes were fingerprinted and their patterns also indicate extreme dissimilarity. By comparing the N-terminal serine and pyridoxal phosphate contents as well as the number of ninhydrin-positive tryptophan and histidine spots of the fingerprints to its assigned molecular weight, it is concluded that the mitochondrial aspartate transaminase is a dimer made up of two very similar monomers. The significance of these findings in relation to the structure of the supernatant isozyme and their possible genetic implications is discussed.

After Fleischer *et al.* (1960) reported the separation of two isozymes of glutamate aspartate transaminase (L-aspartate:2-oxoglutarate aminotransferase EC 2.6.1.1.) from dog heart, the electrophoretically faster migrating enzyme was localized in the supernatant fraction while the slow one was associated with the mitochondrial fraction (Boyd, 1961). Later experiments led to the purification to varying degrees of the two enzymes from a variety of mammalian tissues including rat liver (Boyd, 1966), beef heart and liver, and pig heart (Wada and Morino, 1964). It is well established that these transaminases exist in two distinct forms or isozymes that are easily distinguishable immunologically (Wada and Morino, 1964) by starch gel electrophoresis (Martinez-Carrion *et al.*, 1965) and by differences in their substrate K_m's and rate dependence on pH (Fleischer *et al.*, 1960). In fact, differences in the properties of the two enzymes may result from distinct structural compositions dictated by variations in the genetic code of the particular isozyme. In addition, it is known (Martinez-Carrion *et al.*, 1965) that the S-GAT[1] can exist and be isolated in at least four forms that have similar primary structure and catalytic properties. Therefore, it would be of interest to determine to what extent we can differentiate between

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[1] Abbreviations used: supernatant glutamate–aspartate transaminase, S-GAT; mitochondrial glutamate–aspartate transaminase, M-GAT; *N*-bromosuccinimide, NBS; mercuribenzoate, MB.

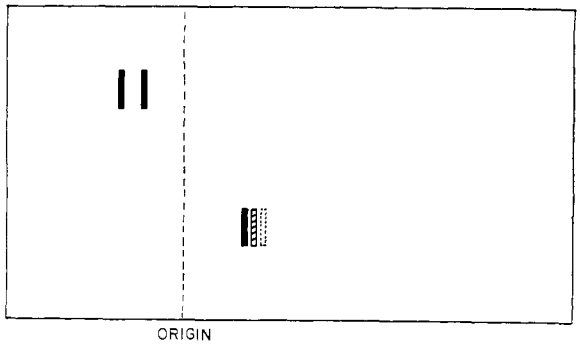

FIGURE 1: Nigrosin stain in starch gel electrophoresis of purified pig heart glutamate aspartate transaminases; (top) mitochondrial isozyme and (bottom) supernatant isozyme. The anode is to the right.

the two isozymes and their multiple forms.

The heart GAT constitutes a unique system for studying isozymes structure and function, inasmuch as all forms of the transaminase carry a prosthetic group (pyridoxal phosphate) that can act as an indicator of events at the active site during enzyme catalysis (Jenkins and D'Ari, 1966). Hence, it is well suited for comparative studies of the catalytic properties of each isozyme and of the multiple forms associated with each isozyme. Since both the mitochondrial and supernatant GAT's can be isolated in reasonable amounts (Martinez-Carrion *et al.*, 1965; Wada and Morino, 1964), it was plausible to undertake studies of their structures, in general, to further the investigation of transaminase catalysis, and in particular, to correlate the genetic and functional relationship of these two transaminases. This work presents a comparative study of the absorption spectra and electrophoretic properties of highly purified mitochondrial and S-GAT, their amino acid compositions, N-terminal amino acids, and peptide maps.

Experimental Procedures and Results

Enzyme Purification. Both transaminases were isolated and purified from pig hearts: the supernatant α-GAT, according to our previous procedure (Martinez-Carrion *et al.*, 1965); the M-GAT, essentially by the method of Wada and Morino (1964) except that the heat step was carried out in 0.15 M sodium succinate buffer instead of sodium maleate. The latter preparation after the hydroxylapatite chromatography was still contaminated by heme-containing proteins and, consequently, was unsuitable for spectral studies. The enzyme preparation was dialyzed against water, adjusted to 0.02 M Tris buffer (pH 8.2), and then added to a 2 × 40 cm column of DEAE-Sephadex A-50 (Pharmacia) previously equilibrated with the same buffer. The red material remained adsorbed to the top of the column while M-GAT was washed through with the 0.02 M Tris. This enzyme was concentrated by ultrafiltration to 5 mg/ml and, when stored at 5°, was stable for several weeks.

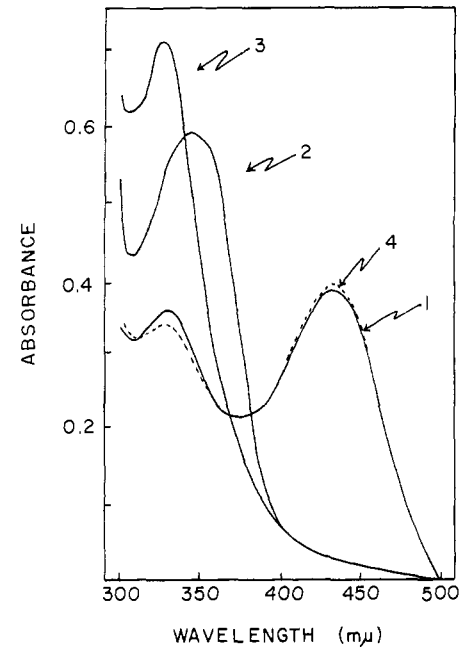

FIGURE 2: Effect of α-ketoglutarate and sodium borohydride on the spectrum of mitochondrial glutamate aspartate transaminase (3.38 mg/ml). Curve 1: enzyme in 0.1 M sodium acetate (pH 5.33). Curve 2: enzyme in 0.05 M Tris (pH 8.2). Curve 3: enzyme after reduction with sodium borohydride and dialysis against 0.05 M sodium acetate or 0.05 M Tris (pH 8.2). Curve 4: enzyme in 0.1 M sodium acetate plus 2.5 mM α-ketoglutarate.

Activity was measured by the direct spectrophotometric method of Lis (1958) at 25° in 0.05 M potassium phosphate buffer at pH values 6 and 7.5. Units of activity were defined as micromoles of oxaloacetate produced per minute per milligram of protein. Protein concentration was followed by measuring the ultraviolet absorption at 280 mμ in buffer solution, after a weight standard was established by extensively dialyzing an aliquot of enzyme against glass-distilled water and then drying the sample to constant weight.

Starch Gel Electrophoresis. The chosen method was our previously published procedure (Martinez-Carrion and Jenkins, 1965). After each electrophoretic separation the gel was sliced horizontally and the upper layer was stained with nigrosin while the lower was tested for GAT activity according to the procedure of Dekker and Rau (1963). The purified enzyme in this system gave at least two distinctive (occasionally three) cationic bands at pH 8.6 (Figure 1). This contrasts the characteristic anionic movement of the multiple forms of the supernatant isozyme. The placement of the nigrosin-staining bands was identical with that of the direct enzymatic assay on the starch gel itself.

Pyridoxal Phosphate Content. Two samples were analyzed by the phenylhydrazine method of Wada and Snell (1961). The readings were made against a standard

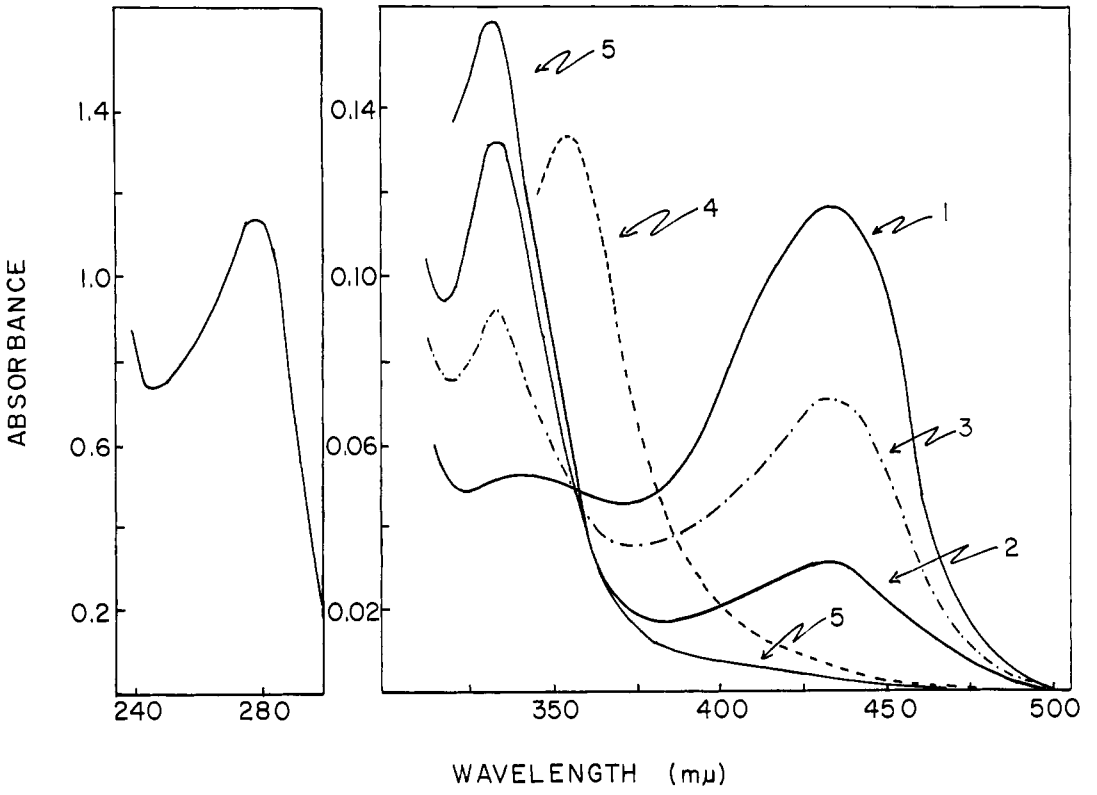

FIGURE 3: Effects of substrates on the spectrum of mitochondrial glutamate aspartate transaminase (0.74 mg/ml). Curve 1: enzyme in 0.1 M sodium acetate (pH 5.0). Curve 2: enzyme in 0.1 M sodium acetate (pH 5.0) plus 7 mM L-glutamate. Curve 3: enzyme in 0.1 M sodium acetate (pH 5.0) plus 30 mM L-glutamate and 0.5 mM α-ketoglutarate. Curve 4: enzyme in 0.05 M Tris (pH 8.2). Curve 5: enzyme in either 0.05 M Tris (pH 8.2) or 0.1 M sodium acetate (pH 5.0) plus 0.1 mM cysteine sulfinic. Absorbance at 280 mμ is identical in all cases.

curve of pure pyridoxal phosphate (Calbiochem), the average coenzyme content being 2.30 ± 0.3 μmoles/μmole of enzyme, assuming a molecular weight of 100,000 (Wada and Morino, 1964).

Absorption spectra were obtained using a Beckman DB-G or a Cary Model 14 recording spectrophotometers. The pig heart M-GAT spectrum resembles that of the supernatant pig heart transaminase in that it absorbs light in the wavelength range of 300–500 mμ. At low pH (pH 5) one absorption maximum appeared at 430 mμ which can probably be ascribed, as in the supernatant enzyme, to a phosphopyridoxal–aldimine enzyme complex, and a second at 335 mμ, that in the supernatant isozyme has been associated with its catalytically inactive γ form (Martinez-Carrion *et al.*, 1965). The ratio of extinctions of these two maxima seem to vary with each preparation (Figures 2 and 3). At high pH (above 7.5) there is only one absorption maximum and that at 355 mμ instead of the 362-mμ value reported for the supernatant enzyme. Assuming that there are two moles of bound pyridoxal phosphate per mole of enzyme, the molar absorbancy coefficient of the bound vitamin B_6 derivative is 8050 ± 300 at 430 mμ and 8350 ± 700 at 355 mμ, these being average values for three enzyme preparations. The $A_{280}:A_{355}$ ratio was 8.7 in Tris buffer (pH 8.2) (Figure 3).

Spectral shifts were observed in the presence of substrates. L-Glutamate or cysteine sulfinic aliquots decreased the absorption maxima at 355 (high pH) and 430 mμ (low pH) with a proportionate increase at 330 mμ. α-Ketoglutarate reversed this shift, the final relative absorbances depending on the ratio of keto acid to amino acid (Figure 3). Since addition of α-ketoglutarate alone to a final concentration of 2.5 M does not seem to significantly change the absorption spectrum of the initial enzyme (Figure 2), it must be concluded that all these initial maxima are due to the pyridoxal form. They do not represent a mixture of pyridoxal and pyridoxamine forms of the enzyme. When L-glutamate either alone or in the presence of α-ketoglutarate was added to the enzyme at high pH and the differential spectrum was recorded, it was observed that the amino acid produced a marked decrease in absorbance at 360 mμ with a proportionate increase at 330 mμ (Figure 4). These changes, like those observed in similar experiments at low pH (Figure 3), also show a reversal by the addition of keto acid. It can be seen from Figures 3 and 4 that addition of the keto–amino acid pairs does not produce enzyme–

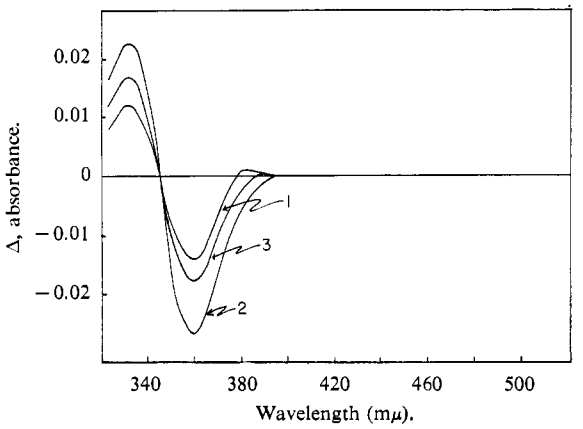

FIGURE 4: Difference spectra of the enzyme (0.42 mg/ml) in 0.05 M Tris (pH 8.2) and of the same enzyme solution in presence of substrates. Curve 1: enzyme in 0.5 mM L-glutamate. Curve 2: enzyme in 5 mM L-glutamate. Curve 3: enzyme in 5 mM L-glutamate plus 0.1 mM α-ketoglutarate.

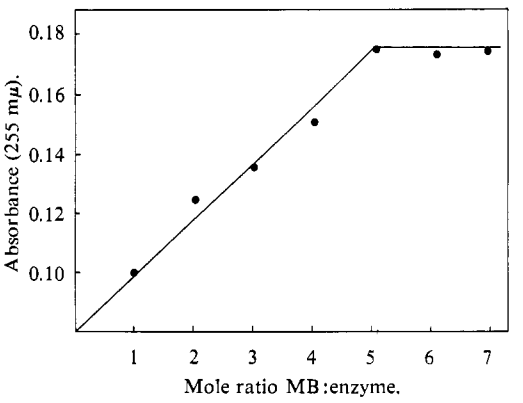

FIGURE 5: Titration of mitochondrial glutamate aspartate transaminase with MB.

substrate complexes absorbing at wavelengths other than 330 or 360 mμ, therefore, eliminating the possibility of formation of a uniquely absorbing binary (of the order enzyme–amino acid or enzyme–keto acid) or ternary complexes (of the order enzyme–amino–keto acid). Furthermore, the results in Figure 4 indicate that M-GAT like the supernatant transaminase could have absorbance at 360 mμ due to the aldimine-linked pyridoxal phosphate and not at 350 mμ as reported by Morino and Wada (1963) or at 355 mμ as shown in Figure 3. Although there is always the possibility that a small shift away from the isosbestic point could be expected from a difference spectrum. Nevertheless, the apparent maximum at the lower wavelength could be due to summation masking by excess of 330-mμ absorbing material that is also insensitive to pH changes.

Reduction of Bound Pyridoxal Phosphate by Sodium Borohydride. A solution containing 10 mg of enzyme/ml in 0.05 M sodium acetate buffer (pH 5.33) was treated with 0.005 M sodium borohydride for 10 min by the dialysis method of Matsuo and Greenberg (1959). This borohydride reduction caused an immediate change in the spectrum. The 430- (low pH) or 355-mμ (high pH) absorbance disappeared, and there was a corresponding increase in the absorption at 330 mμ (Figure 3). Similar spectral shifts have been observed with all pyridoxal phosphate enzymes so far investigated after treatment with sodium borohydride. M-GAT loses all catalytic activity after reduction with sodium borohydride and subsequent dialysis for 10 hr against 0.05 M potassium phosphate buffer (pH 7.0). We believe that, as in S-GAT and other pyridoxal phosphate proteins, sodium borohydride reduces to a secondary amine the azomethine linkage between the formyl group of pyridoxal phosphate and an ε-amino group of a lysyl residue in the protein.

Amino Acid Analysis. All analyses were performed with a Technicon Automatic AutoAnalyzer. Duplicate samples (0.9 mg) were hydrolyzed for 16, 41, and 72 hr in 6 N HCl at 110° in evacuated sealed tubes. Six separate analyses of acid-hydrolyzed mitochondrial enzyme were made and the results are shown in Table I. The values of serine and threonine were extrapolated to zero time to take account of the decomposition of these amino acids during acid hydrolysis, whereas those values for valine and isoleucine were the ones at the longest hydrolysis time to account for the slow hydrolysis of peptides of these amino acids.

Cysteine was determined as cysteic acid after oxidation of the sample with performic acid, hydrolysis for 20 hr in 6 N HCl at 110°, and separation of the cysteic acid by ion-exchange chromatography (Schram *et al.*, 1954) (Calbiochem cysteic acid used as standard). Tryptophan content was measured by the direct spectrophotometric method of Bencze and Schmidt (1957) and titration with NBS (Pierce Chemical) (Patchornik *et al.*, 1958). For the latter, the reaction mixture contained 0.1 M sodium acetate buffer (pH 4.4) and amounts of protein (0.37 mg/ml). Aliquots of 0.01 M NBS solution were added at intervals, and readings were taken at 280 mμ against a blank containing the same mixture without NBS. Three different titrations with NBS gave results ranging from 9.5 to 10.6 residues/50,000 mol wt. We have adopted an average value for measurements made with both methods of ten residues per monomer of M-GAT. From Table I, it can be seen that there is a marked difference in the amino acid composition of the two isozymes.

Titration of M-GAT with MB. M-GAT was titrated spectrophotometrically with MB by the method of Boyer (1954). A fixed concentration of enzyme (13.4 μM, mol wt 50,000) was treated with 1–8 equiv of MB in 0.1 M sodium acetate buffer (pH 5) at 25° and the absorbance was followed up to a constant value. Figure 5 indicates that our enzyme preparation contains approximately five titratable SH groups per molecule of monomer. This value was the same for measurements made in the presence of 4 M urea, and it is the same as that one

TABLE I: Amino Acid Compositions of Mitochondrial and Supernatant Glutamate Aspartate Transaminase.[a]

Amino Acid	Amino Acid Residues (g/100 g of protein)	Calcd Residues for Mol Wt 50,000	Residues to nearest Integer	
			M-GAT	S-GAT[a]
Arginine	9.19 ± 0.08	21.3	21	26
Histidine	3.32 ± 0.04	12.1	12	8
Lysine	7.99 ± 0.12	31.1	31	20
Phenylalanine	5.85 ± 0.04	19.6	20	23
Tyrosine[b]	3.92 ± 0.05	12.1	12	12
Leucine	6.89 ± 0.07	30.4	30	40
Isoleucine	6.26 ± 0.06	27.7	28	18
Methionine	3.26 ± 0.03	12.4	12	6
Half-cystine[c]			5	5
Valine	5.66 ± 0.06	28.2	28	29
Alanine	5.30 ± 0.05	37.4	37	33
Glycine	4.10 ± 0.04	36.0	36	28
Proline	3.65 ± 0.02	18.8	19	24
Glutamate	11.00 ± 0.03	42.7	43	46
Serine[b]	3.90	22.3	22	26
Threonine[b]	3.25	16.1	16	24
Asparatate	9.11 ± 0.04	39.7	40	42
Tryptophan[d]			10	10

[a] Data from Martinez-Carrion *et al.* (1967). [b] Extrapolated to 0-hr hydrolysis. [c] As cysteic acid after performic acid oxidation. [d] Spectrophotometric method of Bencze and Schmidt (1957). [e] Hydrolysis times are 20, 41, and 72 hr at 110°.

reported for S-GAT (Turano *et al.*, 1963) under identical conditions.

Amino-Terminal Analysis. Dinitrophenylation and subsequent hydrolysis of the mitochondrial transaminase (0.1 μmole) was carried out by a modification of Sanger's procedure (Fraenkel-Conrat *et al.*, 1955). The hydrolyzed sample was extracted to separate the acid and basic amino acids and the paper chromatography was performed as indicated by Fraenkel-Conrat *et al.* (1955). Only one spot due to a DNP-amino acid was obtained from the ether-soluble components; and none, except the anticipated DNP-lysine, -tyrosine, and -histidine were found among the water-soluble derivatives. The spot in the ether phase was identified as DNP-serine by its chromatographic mobility with known DNP-amino acid derivatives (Sigma Chemical). The DNP-serine spot was cut and eluted with 1% $NaHCO_3$ from the two-dimensional chromatogram and its spectrum was recorded. By using the extinction coefficient of DNP-serine at 360 mμ (Fraenkel-Conrat *et al.*, 1955), 1 residue of DNP-serine residue/48,000 ± 3300 of protein was calculated. Correction for losses of DNP-serine during hydrolysis, chromatography, and manipulation was done by adding 0.1 μmole of synthetic DNP-serine to 0.1 μmole of dinitrophenylated enzyme prior to acid hydrolysis. The extracted DNP-serine derivatives were further identified by thin layer chromatography in silica gel with chloroform–methanol–acetic acid (95:5:1) as solvent. Turano *et al.* (1963) previously reported L-alanine as the N-terminal amino acid in S-GAT isozyme.

Peptide Maps. To 10-mg samples of the mitochondrial and supernatant enzyme in 0.005 M acetate buffer (pH 5), 2 mg of sodium borohydride was added and the solutions were immediately centrifuged to prevent foaming. After 30 min at room temperature the pH was adjusted to 1 by addition of 3 N HCl. The resulting cloudy suspension was immediately boiled for 3 min to ensure complete protein denaturation. The precipitate was removed by centrifugation and washed twice with 2 ml of 0.05 M ammonium bicarbonate buffer (pH 8.5). A suspension of the denatured protein in 1 ml of the same buffer was digested for 24 hr at 37° by 0.5 mg of trypsin (Calbiochem). A small amount of insoluble material remained in the mitochondrial enzyme but none in the cytoplasmic. The insoluble material was removed by centrifugation and the supernatant solutions were brought to dryness in a flash rotary evaporator. The dried material was recovered in 0.2 ml of water and aliquots were spotted on Whatman 3MM paper (94 × 45 cm) for electrophoresis at pH 4.6 in a pyridine–acetic acid–water (25:25:2950) buffer. The electrophoresis was carried out in a Savant Instruments, Inc., high-voltage paper electrophoresis apparatus using a potential of 45 v/cm for 1.20 hr. The second dimension was developed by descending chromatography in a solvent of butanol–pyridine–acetic acid–water (15:10:3:12). After drying, the maps were

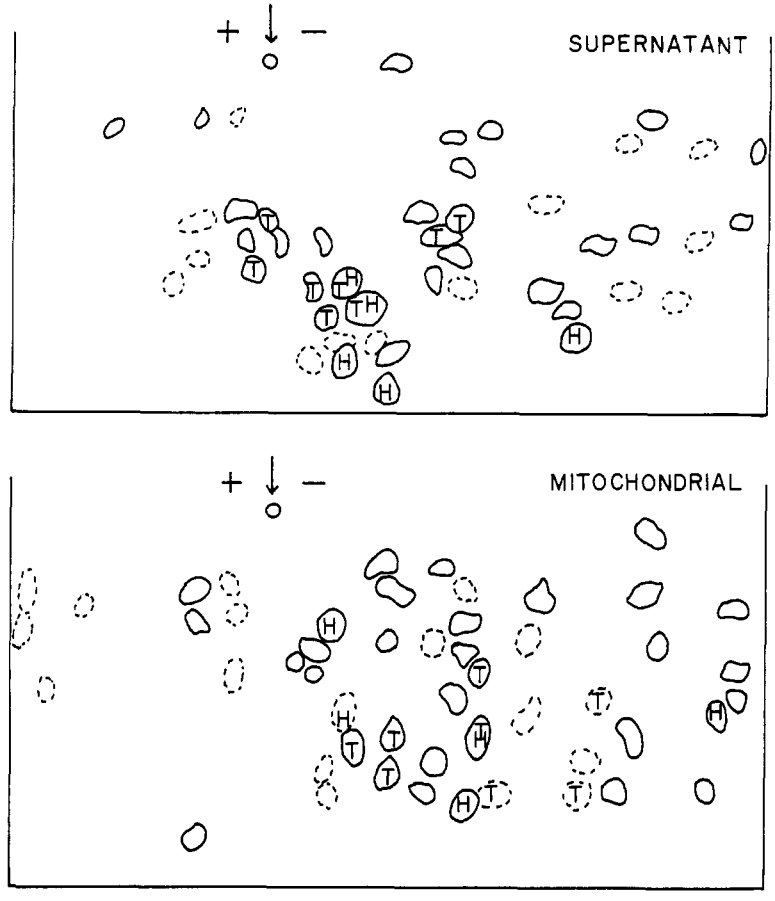

FIGURE 6: Peptide maps of tryptic digest of pig heart mitochondrial and supernatant glutamate aspartate transaminase. Histidine-containing peptides are marked H; tryptophan-containing peptides, T. Solid tracings correspond to highly colored ninhydrin-positive spots. Dotted tracings refer to weakly colored ninhydrin-positive spots.

stained by dipping in a 0.5% ninhydrin–acetone solution and the color was developed by heating in an oven at 60° for 20 min. Ehrlich's stain for tryptophan (Smith, 1960) was applied directly to ninhydrin-treated fingerprints. Staining for histidine-containing peptides was carried out by dipping in the Pauli reagent (Smith, 1960). Figure 6 shows the distribution of tryptic peptides after staining with ninhydrin. The results after the staining of the same maps with the reagents specific for typtophan- and histidine-containing peptides are also shown on the same fingerprints (Figure 6). A total of 51 and 46 ninhydrin-positive peptides was identified for the M- and S-GAT, respectively, and eight and eight tryptophan spots and five and five histidine spots. While the peptide distribution was reproducible with several enzyme preparations, the test for tryptophan- and histidine-containing peptides was done only once for each isozyme. The consistent variance of mitochondrial and supernatant peptide maps indicates the dissimilarity of many peptides in the enzymes. The number of ninhydrin-positive spots, a summation of the number of lysine and arginine residues in the protein from a tryptic digestion, and the number of tryptophan and histidine spots were always one-half or less than one-half of the values determined from our amino acid analysis based on a reported molecular weight of 100,000 (Wada and Morino, 1964). This feature, common to the mitochondrial and supernatant enzymes, suggests that the former, like the latter, is a dimer consisting of two subunits of identical or very similar structure. These observations agree with the fact that M-GAT possesses two N-terminal serine residues and two pyridoxal phosphate groups for a protein with a molecular weight of 100,000.

Discussion

The spectral data of the mitochondrial pig heart isozyme conforms to the previously reported spectral differences in the beef liver GAT isozymes (Morino and Wada, 1963). It is worth noting, however, that there is no difference in the absorption spectrum between the two heart isozymes if the maxima recorded in the difference spectrum of M-GAT against M-GAT in presence of substrates are considered (Figure 4). If the reason for an apparent maximum at 355 mμ instead of 360 mμ is masking by excessive 330-mμ absorbing material, it is then necessary to explain the nature of this latter material. By all indications, this is due to enzyme-bound

pyridoxal phosphate because addition of NaOH to M-GAT to a final concentration of 0.1 N results in the disappearance of the 330-mμ maximum as well as the one at 355 mμ and the appearance of one corresponding to free pyridoxal phosphate (in basic solution) with absorbance at 390 mμ. Using the molar absortivity values for the latter maximum we can account for all the pyridoxal phosphate present in the enzyme by comparison with the results obtained with the phenylhydrazine method. Therefore, it is probable that the 330-mμ absorbing material is due to unspecifically bound pyridoxal phosphate with no catalytic role. Since it is known that pyridoxal phosphate binds to lysine residues of proteins (Dempsey and Christensen, 1962), it is most likely that this vitamin B_6 derivative becomes bound to the enzyme during heating of M-GAT in the presence of free pyridoxal phosphate (Morino and Wada, 1963) in the purification procedure. In support of our hypothesis, is the observation that, although we obtain a pyridoxal phosphate content of 2 μmoles/μmole of enzyme with our best preparations, it was observed that those enzymes preparations with high 330-mμ absorbing material had the same specific activity but yielded values as high as 2.6 μmoles of pyridoxal phosphate/μmole of enzyme.

The ratio of absorbance owing to protein at 280 mμ and that owing to bound pyridoxal phosphate can only be compared with our previous results for pig heart S-GAT. And, in both cases, they are nearly identical. No values are available for other M-GAT's. The pig heart M-GAT, like the supernatant one, can react with cysteine sulfinic and its substrates L-glutamate or L-aspartate but not with their D isomers. These reactions result in a shift in the absorbance due to the bound pyridoxal phosphate from an aldimine-type link with maxima at 355 or 435 mμ at high or low pH to a form absorbing at 333 mμ that is normally ascribed to a pyridoxamine phosphate-enzyme complex.

Our data also support the view that M-GAT is the isozyme that migrates to the cathode at pH 8.6 in starch gel electrophoresis and that, as reported by us in crude extracts (Martinez-Carrion *et al.*, 1967), it occurs in at least two forms in the purified state. The separation of these multiple forms of M-GAT, currently being studied in our laboratory, is the prerequisite for studying similarities and dissimilarities between the multiple forms.

Pig heart GAT isozymes differ in electrophoretic properties mainly because of structural differences as evidenced by their distinct amino acid composition and peptide maps. Both enzymes, however, possess the same content of tyrosine and tryptophan residues which accounts for their similar molecular extinction coefficients at 280 mμ. Further, they are similar in their cysteine content and all their sulfhydryl groups appear to be free and in the reduced state as judged by their ease in combining with MB. The isozymes of GAT resemble each other in their size and in the fact that each consists of two similar polypeptide chains. But the supernatant monomer has alanine as the N-terminal amino acid while, for the mitochondrial monomer, it is serine. Therefore, if, according to current concepts, protein synthesis is initiated at the amino end of the polypeptide chain (Dintzis, 1961), they should have different initiators in the coding DNA molecule. The GAT isozyme system is one more example of proteins with basically the same catalytic properties but quite different structures. Since their sites of cellular localization are different, it would be interesting, indeed, to know if the sites of synthesis are also different.

As considerable genetic evidence exists that some of the mitochondrial proteins are determined by nonchromosomal genes, as well as the presence of DNA in some mitochondria, as recently reviewed by Horowitz and Metzenberg (1965). It is possible that the M-GAT is one protein that receives information and is assembled in this subcellular entity, while the supernatant enzyme follows the "standard" pathway receiving its information from nuclear chromosomal DNA, with translation to and assembly on the cytoplasmic ribosomes. Regardless of the mechanism and the site of synthesis it is evident that both enzymes cannot be coded by the same gene or by a slightly altered form of the same gene. In conclusion, it is worth noting that the other key enzyme in the metabolic fate of oxaloacetate, malic dehydrogenase, is known to exist in forms localized in the mitochondria and the supernatant fraction, structurally different forms with dissimilar substrate K_m's (Thorne, 1962; Thorne and Cooper, 1963).

Acknowledgments

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Tripeptide (Glutathione) Synthetase. Purification, Properties, and Mechanism of Action*

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ABSTRACT: Tripeptide (glutathione, γ-glutamyl-α-aminobutyrylglycine) synthetase has been purified about 5000-fold from Baker's yeast; the enzyme is homogeneous by electrophoretic and ultracentrifugal criteria (sedimentation coefficient 6.1 S, mol wt 123,000). Various properties of the enzyme including its amino acid composition, kinetic behavior, and specificity have been studied. The ability of the enzyme to catalyze adenosine triphosphate–adenosine diphosphate (ATP–ADP) exchange decreases greatly during purification. Evidence has been obtained that the rate of formation of the enzyme-bound dipeptide intermediate (γ-glutamyl-α-aminobutyryl phosphate) in the absence of acceptor is of the same order as that of the over-all reaction, and that the ADP formed in this reaction dissociates relatively slowly from the enzyme. It is tentatively concluded there are about four active sites per molecule of enzyme.

Previous studies in this laboratory have provided evidence that the enzymatic synthesis of tripeptides [glutathione, ophthalmic acid (γ-glutamyl-α-aminobutyrylglycine)], which takes place according to eq 1,

$$\gamma\text{-glutamylamino acid} + \text{glycine} + \text{ATP}^{1} \underset{Mg^{2+}}{\overset{\text{synthetase}}{\rightleftharpoons}} \text{tripeptide} + \text{ADP} + P_i \quad (1)$$

involves the intermediate formation of an enzyme-bound carboxyl-activated dipeptide derivative, *i.e.*, γ-glutamylaminoacyl phosphate. Pulse-labeling experiments provided the initial evidence for such an intermediate; subsequently a compound with the properties of γ-glutamyl-α-aminobutyryl phosphate was isolated from reaction mixtures containing the yeast synthetase, γ-glutamyl-α-aminobutyrate, ATP, and magnesium ions (Nishimura *et al.*, 1963). It was later found that the enzyme could utilize chemically synthesized γ-glutamyl-α-aminobutyryl phosphate for both the synthesis of ATP and of γ-glutamyl-α-aminobutyrylglycine (Nishimura *et al.*, 1964).

The present studies were undertaken in an effort to purify the enzyme further and to obtain additional information about its properties and the mechanism of the reaction. In the course of this work a preparation of the enzyme from yeast has been achieved which is essentially homogeneous by electrophoretic and ultracentrifugal criteria. Various properties of the enzyme, including its molecular weight and amino acid composition, have been determined. Further

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[1] Abbreviations used: ADP and ATP, adenosine di- and triphosphates; DPNH, reduced diphosphopyridine nucleotide.