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Conformational Properties of the Isoenzymes of Aspartate Transaminase and the Enzyme–Substrate Complexes*

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ABSTRACT: Possible variations in conformation of two pig heart glutamate aspartate transaminase isozymes, mitochondrial and supernatant, with the elimination of coenzyme or the binding of substrates, were investigated using optical rotatory dispersion, circular dichroism, and microcomplement fixation. The multiple forms, α, β, and γ, within the supernatant isozyme are identical in dichroicity in the ultraviolet region. The multiple forms, A, B, and C, of the mitochondrial isozyme are also indistinguishable from each other and from their apoenzymes. Both enzyme groups have as much as 37–40% α-helix content but none of the subforms in the mitochondrial isozyme react immunologically with subforms in the supernatant isozyme. In the visible region, the circular dichroism patterns for the optically active bands of both isozymes are identical. Although the amplitude of these bands or Cotton effects centered at 430 or 360 mμ vary in absolute magnitude for each subform within an isozyme set, each is proportional to its absorbance. The species of enzyme-bound pyridoxal phosphate absorbing at 340 mμ shows very little optical activity. In the 250–300-mμ range, the apoenzymes of both isozymes show positive dichroic bands. The positions of these bands vary in each isozyme group and some of the optically active transitions change to negative dichroicity upon binding of pyridoxal phosphate. The new bands are centered at 298 and 290 mμ in the supernatant isozyme and at 290 mμ in the mitochondrial isozyme.

The overall circular dichroism pattern in the far-ultraviolet region of the two isozyme groups is unaffected by substrates or changes in pH. L-Aspartate changes the enzyme's absorption spectra to give maxima at 492, 430, and 330 mμ in the supernatant isozyme and 430 and 330 mμ in the mitochondrial isozyme. Only those species with absorbance at 330 mμ show appreciable positive ellipticity but have diverse dissymmetry factors, $\Delta\epsilon/\epsilon$, for each isozyme group. *erythro*-β-Hydroxyaspartate binds to each isozyme forming a characteristic semiquinoid-type complex with absorbance at 492 mμ (supernatant isozyme) or 498 mμ (mitochondrial isozyme) and another maximum in the 330-mμ region. They appear as negative circular dichroism bands at 490–500 mμ and a positive band in the 330-mμ region. The substrate analog, α-methylaspartate, also binds to the active site, producing species absorbing at 430 and 360 mμ. Only the 360-mμ species is optically active and shows a positive dichroic band in both isozymes. Binding of substrates or their analogs alters the optical properties of aromatic chromophores in the supernatant isozyme but not those in the mitochondrial isozyme. An interpretation consistent with the mechanism of transamination is offered in which interactions of the active center with the coenzyme and substrates can be detected and accounted for by conformational changes of the substrate in the covalent pyridoxal phosphate–substrate complex and protein residues at the active site.

The supernatant isozyme and mitochondrial isozyme, although distinct in primary structure (Martinez-Carrion, M., and Tiemeier, D. (1967), *Biochemistry 6*, 1715), have equivalent amounts of ordered structure, mostly as α helix. The active-site topology is very similar in both.

Aspartate transaminase (EC 2.6.1.1) exists as distinctive isozymes with specific cytological localization (Martinez-Carrion *et al.*, 1967). This isozyme system consists of two groups of chemically distinct proteins (Martinez-Carrion and Tiemeier, 1967), each possessing multiple forms with similar structural properties (Martinez-Carrion *et al.*, 1967; Michuda and Martinez-Carrion, 1969a). However, little is known about their conformational properties.

* From the Chemistry Department, Biochemistry and Biophysics Program, University of Notre Dame, Notre Dame, Indiana 46556. *Received October 27, 1969.* Supported by a grant from the National Institutes of Health (HE-11448).

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These isozymes, like most phosphopyridoxal enzymes, form unique enzyme–substrate complexes with characteristic optical properties. The supernatant isozyme group has only been subjected to conformational studies by spectropolarimetric methods in restricted regions of the spectrum (Fasella and Hammes, 1964, 1965; Ivanov *et al.*, 1967) and even less is known about the conformation of the mitochondrial isozyme (Michuda and Martinez-Carrion, 1969a).

The existence of two structurally distinct isozyme groups poses major questions on the conformation of the isozymes and their multiple forms.

An approach to probing some of the conformational parameters is the use of both circular dichroism and microcomplement fixation techniques. Of specific interest to this study are the following: (a) How do the far-ultraviolet regions of the circular dichroism spectrum compare for each subform of the supernatant and mitochondrial groups? (b) Does the binding of pyridoxal phosphate to each subform of the two main isozymes produce comparable dichroic effects in the visible region of the spectrum? (c) Do substrates or substrate analogs affect the circular dichroism spectrum in the ultraviolet region? (d) Are there discrepancies in how substrates or their analogs affect the dichroicity of bound pyridoxal phosphate in each isozyme group? (e) Can the multiple forms within each isozyme be distinguished by the sensitive microcomplement fixation technique?

Experimental Procedure

Chemicals. α-Methylaspartate was purchased from Sigma Chemical Co. Other amino acids and keto acids were A grade samples from Calbiochem. Guanidine hydrochloride was Spectral Grade quality from Mann Research Laboratories. Sheep hemolysin, sheep red blood cells, and guinea pig complement were obtained from Colorado Serum Co.

Enzyme Preparations. Purification of the supernatant and mitochondrial isozymes and the fractionation of the multiple forms has been described elsewhere (Martinez-Carrion *et al.*, 1967; Michuda and Martinez-Carrion, 1969a). Protein concentrations were calculated from the optical densities at 280 mμ. Preparation of the apoenzymes of supernatant glutamate aspartate transaminase was accomplished by adding to 1 ml of enzyme (5–10 mg) in 0.005 M potassium phosphate buffer (pH 7.5) a molar excess of cysteinesulfinate. This was followed by 1 ml of 1 M monobasic potassium phosphate. The mixture was then incubated at 25° for 10 min and solid, finely ground, ammonium sulfate was added to 70% saturation. The precipitate was collected by centrifugation and recovered in 1 ml of 0.005 M potassium phosphate buffer (pH 7.5). The entire process was repeated and the enzyme dialyzed against 0.1 M potassium phosphate buffer (pH 7.5). This procedure gives 85–90% recovery of enzyme which is over 95% free of pyridoxal phosphate. The specific activity can be restored by addition to 100 μM of pyridoxal phosphate and removal of the excess pyridoxal phosphate by dialysis. Apoenzyme of the mitochondrial isozyme was prepared by the method of Michuda and Martinez-Carrion (1969a). Isozymes in the pyridoxamine form were prepared by the method of Jenkins and D'Ari (1966a).

Spectra. Absorption spectra were recorded on a Cary Model 15 recording spectrophotometer. When comparing absorption, and circular dichroism spectra, the same samples were used at 25° and under the conditions given in each figure. Absorbance is expressed in optical density units.

Optical Activity. Circular dichroism measurements were made with a Cary Model 60 spectropolarimeter equipped with a circular dichroism attachment and set for a half-band width of 15 Å. Circular dichroism values were measured to $\pm 0.0005°$ from the visible to 230 mμ, to $\pm 0.002°$ from 230 to 205 mμ, and to ± 0.02 below this region. Wavelength readings were reproducible in all spectral measurements to ± 0.5 mμ. Molecular ellipticity values, $[\theta]$, were calculated by using the expression $[\theta] = (\theta/10)m/lC'$, where m is the mean residue molecular weight of the sample, l the path length in the sample solution in cm, and C' is the concentration in g/cm^3. $[\theta]$ has the dimensions of (deg/cm^2)/dmole of amino acid residue. $\Delta\epsilon$ values were calculated from $\theta°$ (direct values from the instrument) using the relationship $\theta° = 33\Delta\epsilon$. $[\theta']$ include the Lorentz correction for refractive indices. ϵ's are reported as absorbances at the specified wavelengths for 1-cm light paths. In all experiments with amino acids, the racemic mixtures were employed to eliminate rotations due to substrate.

Microcomplement Fixation. Female California white rabbits were injected intramuscularly with 10 mg of the respective forms of the supernatant and mitochondrial enzymes, blended with complete Freund's adjuvant. After 21 days, 10-ml blood samples were taken. The serum showed antigenic value to double-diffusion experiments (Ouchterlony, 1949). The rabbits were reinjected intraperitoneally with 5 mg of the respective transaminase. One month later they were bled by heart puncture and the serum separated. Studies on these sera were based on the microcomplement fixation technique of Wasserman and Levine (1961).

Results

Circular Dichroism of the Pyridoxal Form of the Holoenzymes. The binding of the pyridoxal phosphate chromophore imparts extrinsic, pH-dependent Cotton effects in the visible region. The effects were previously observed (Fasella and Hammes, 1964; Breusov *et al.*, 1964) in optical rotatory dispersion studies of preparations containing unknown mixtures of the three forms of the supernatant isozyme, α, β, and γ. The amplitude of these Cotton effects can better be studied by circular dichroism since the intrinsic protein rotation does not affect measurements in the visible region. Qualitatively all three multiple forms of the supernatant isozyme show positive dichroic bands that mimic their absorption spectra with maxima at 430 mμ (low pH) and 360 mμ (high pH) as indicated in the representative spectra of Figure 1. Quantitatively the differences in absorbances of α, β, and γ parallel their dissimilarities in dichroicity (Table I) such that dissymmetry $\Delta\epsilon/\epsilon$ values are identical. The absorption band at 340 mμ is almost devoid of any dichroic effect. This absorption band was formerly ascribed to bound, catalytically inert pyridoxal phosphate and is particularly prominent in the γ form which is of low transaminase activity.

Present circular dichroism studies reveal new details in the region of absorbance of the aromatic amino acids in each protein with several optically active transitions appearing as two major negative peaks centered at 298 and 289 mμ and three minor positive peaks at 284, 277, and 270 mμ at pH 5. A rise in pH produces minor shifts in the position of the two

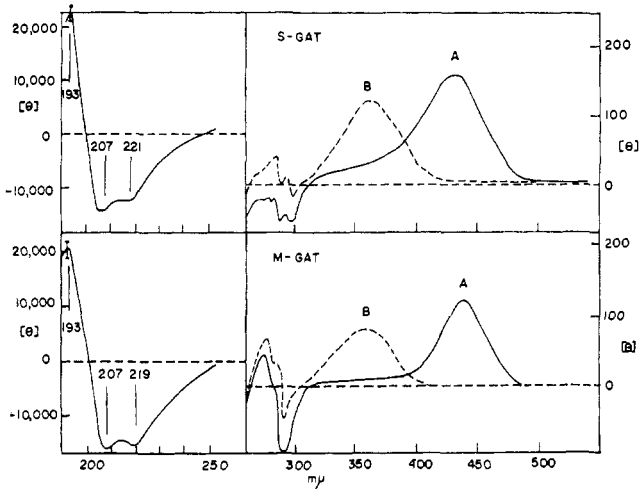


FIGURE 1: Representative circular dichroism of supernatant and mitochondrial isozymes at 25°. (A) (——) 550–260 mμ in 0.05 M sodium acetate (pH 5.1) from 190 to 250 mμ in 0.1 M KCl and the pH was adjusted to 5.1 with dilute HCl. (B) (---) In 0.1 M KCl at pH 8.5 adjusted with dilute NaOH. The specific details about the dichroic properties of the multiple forms of the supernatant and mitochondrial isozymes are summarized in Tables I and II.

major peaks at 300 and 290 mμ and conversion of the minor peaks into a single major positive band with a maximum at 285 mμ and shoulder at 270 mμ (Figure 1).

The A, B, and C subforms of the mitochondrial isozyme show dispersion curves that are remarkably similar to those of their supernatant isozyme (Figure 1). The Cotton effects appear as positive dichroic bands at 436 and 358 mμ, and, like the supernatant isozyme, the species of bound pyridoxal phosphate absorbing at 340 mμ has low dichroicity or none at all (Table II). The aromatic region of the protein spectrum shows two well-defined extrema around 285 mμ. Unlike similar bands in the supernatant enzyme, the positions of these peaks do not vary with pH. However, a rise in pH is followed by a decrease in the amplitude at 290 mμ, accompanied by an increase in the positive dichroic band at 277 mμ. In the far-ultraviolet region the circular dichroism curves show two negative ellipticity bands with extrema at 219 and 207 mμ and a positive band centered at 193 mμ. The dissymmetry factor and location of these ultraviolet bands or dispersion curves are identical for A, B, and C

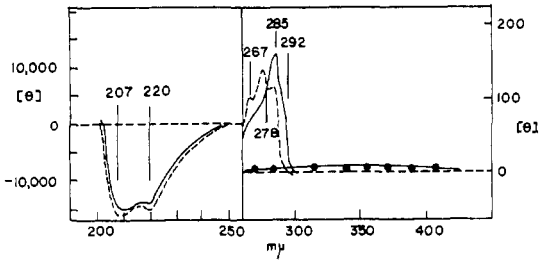


FIGURE 2: Visible and ultraviolet circular dichroism of the apoenzymes of supernatant (——) and mitochondrial glutamate aspartate transaminase (---) at 25° in 0.1 M KCl adjusted to pH 7.0 with dilute NaOH or HCl. Enzyme after 10 hr in 4 M guanidine hydrochloride (●——●).

TABLE I: Visible and Ultraviolet Circular Dichroic Data for the Supernatant Isozyme Multiple Forms and Their Apoenzymes.

Enzyme Subform	λ_{max}	θ	$[\theta]^a$	$(\Delta\epsilon/\epsilon)^b \times 10^3$	$[\theta']$
Holo α	430	0.0178	165	3.37	
	365	0.0132	122	1.92	
	221	0.138	−12,800		−9,760
	207	0.160	−14,800		
	193	0.250	23,150		
Holo β	430	0.0108	100	3.2	
	365	0.0122	113	1.7	
	221	0.145	−13,400		−10,200
	207	0.168	−15,500		
	193	0.250	23,150		
Holo γ	430	0.0080	74	2.98	
	365	0.0056	51.8	1.21	
	340	0.0056	51.8	1.08	
	221	0.145	−13,400		−10,200
	207	0.168	−15,500		
	193	0.250	23,150		
Apo γ	340	0.00158	14.5	0.497	

[a] $[\theta]$ expressed in terms of mean residue weight = 114. [b] $\Delta\epsilon$ calculated from θ values by using $\Delta\epsilon = \theta/330$. ϵ the absorbance at the given concentration. [c] $[\theta']$ includes refractive index correction.

and are unaffected by variations in the pH region from 5 to 9 (Table II).

Apoenzymes. Using optical rotatory dispersion data, conformational differences have been reported between apo- and holoenzymes (Fasella and Hammes, 1964, 1965) with supernatant isozyme preparations which were mixtures of all multiple forms. However, our circular dichroism results indicate that the dispersion curves of the apoenzymes are pH independent and show the same rotation values as those of the holoenzymes for any multiple form in either the supernatant or mitochondrial isozyme (Tables I and II).

The circular dichroism spectra of the apoenzymes show specific changes in the aromatic region of the spectrum (Figure 2). From 250 to 300 mμ there are several transitions with dichroic positive maxima at 285 mμ and shoulders at 292 and 267 mμ for the supernatant isozyme and maxima at 278, 285, and 267 mμ for the mitochondrial isozyme. These transitions are similar in wavelength but opposite in sign to those observed in the holoenzymes and they disappear in the presence of 4 M guanidine hydrochloride (Figures 1 and 2). Below 250 mμ, both isozyme systems show similar curves with negative ellipticity bands with peaks at 220 and 207 mμ, characteristic of the n–π^* and π–π^* amide transitions (Tables I and II).

The circular dichroism of the pyridoxamine enzymes is given in Figure 3. Both isozymes show distinctive ellipticity bands of positive sign centered at 333 mμ, due to bound pyridoxamine phosphate. Below 330 mμ (Figure 3B), the pyridox-

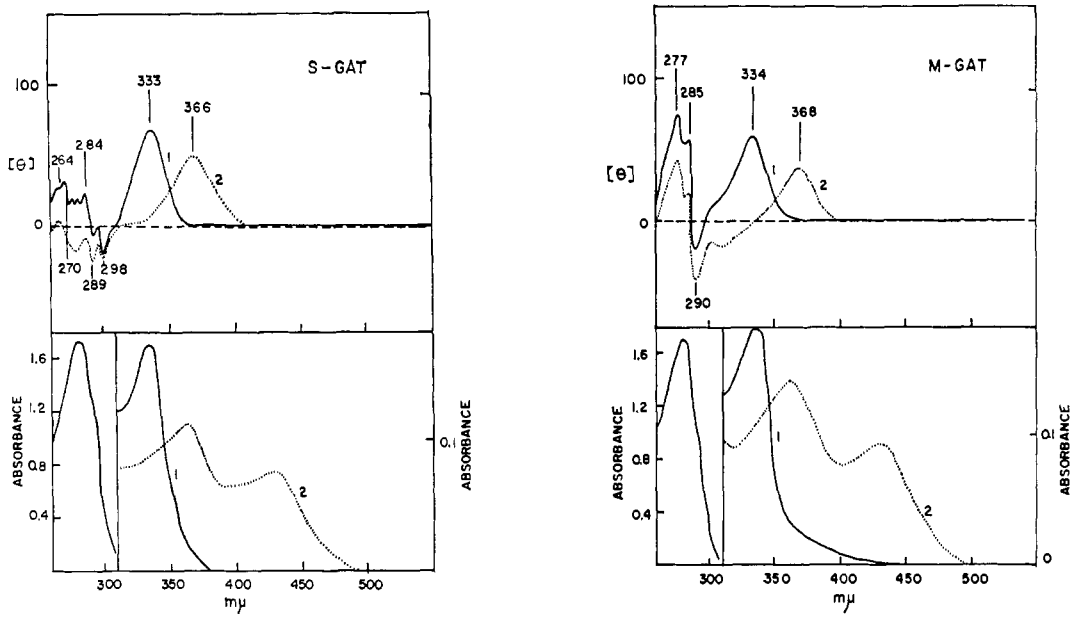


FIGURE 3: Circular dichroism and absorption spectra of the isozymes of glutamate aspartate transaminase at 25°. 1.23-mg/ml enzyme concentration in 0.05 M sodium pyrophosphate buffer (pH 9.0). Top: circular dichroism; Bottom: absorption spectra (A, left) supernatant isozyme, (B, right) mitochondrial isozyme (1) pyridoxamine enzyme; (2) isoenzymes in the presence of 0.01 M DL-α-methylaspartate.

amine form of the mitochondrial isozyme shows the same transitions for the aromatic amino acid chromophores as those of the phosphopyridoxal enzyme under identical experimental conditions: a negative band centered at 290 mμ and two positive bands at 285 and 277 mμ. On the other hand, the supernatant isozyme (Figure 3A) retains only some of the characteristics of the pyridoxal form: negative bands at 298 and 289 mμ and a positive one at 284 mμ. At lower wavelengths there appears a series of positive bands in the 270-mμ region and a shoulder at 264 mμ.

The circular dichroism of the enzyme-substrate complexes of each isozyme are shown with the absorption spectra of these complexes (Figures 3–5). Each isozyme group consisting of a mixture of their multiple forms was studied in the presence of those substrates or pseudosubstrates with which they are known to form distinctive enzyme–substrate complexes (Jenkins and Taylor, 1966; Jenkins and D'Ari, 1966b; Michuda and Martinez-Carrion, 1969b). α-Methylaspartate, *erythro*-β-hydroxyaspartate, and L-aspartate form Schiff's base derivatives with the pyridoxal phosphate chromophore in the enzyme. The absorbances and absorption wavelength of these complexes is unique and has been extremely useful in studying the mechanism of transamination. Since the spectral characteristics of the various complexes are very similar, a study of circular dichroism, both in the visible and ultraviolet region helps in understanding the effects of substrate binding on the conformation near the pyridoxal phosphate and in the overall protein tertiary structure.

α-Methylaspartate forms Schiff's bases with the pyridoxal phosphate in each isozyme, giving species absorbing at 430 and 360 mμ (Figure 3). With this substrate, because of the methyl group on the α carbon of the amino acid, transamination cannot proceed to completion. Species absorbing at 430 mμ are devoid of any optically active transitions but those absorbing around 360 mμ give distinctive positive dichroic bands in each isozyme group. Binding of this amino acid does not produce effects in the aromatic region of the mitochondrial isozyme (Figure 3B). However, it does alter the circular dichroism spectrum of the supernatant isozyme with an increase in the negative amplitude of the 298-mμ band

TABLE II: Visible and Ultraviolet Circular Dichroic Data for the Mitochondrial Isozyme Multiple Forms and Their Apoenzymes.

Enzyme Subform	λ_{max}	θ	$[\theta]^a$	$(\Delta\epsilon/\epsilon)^b \times 10^3$	$[\theta]^c$
Holo A	436	0.0140	130	3.27	
	358	0.00912	84.4	1.7	
	219	0.170	−15,700		−12,000
	207	0.180	−16,700		
Holo B	436	0.0152	141	3.13	
	358	0.0120	111	1.69	
	219	0.160	−14,800		−11,800
	207	0.170	−15,750		
	193	0.230	23,300		
Holo C	436	0.0168	155.5	3.17	
	358	0.0128	118.5	1.73	
	219	−0.167	−15,450		−12,000
	207	−0.180	−16,700		
	193	0.230	21,300		

[a] $[\theta]$ expressed in terms of mean residue weight 114. [b] $\Delta\epsilon$ calculated from θ values by using $\Delta\epsilon = \theta/330$. ϵ the absorbance at the given concentration. [c] $[\theta']$ includes refractive index correction.

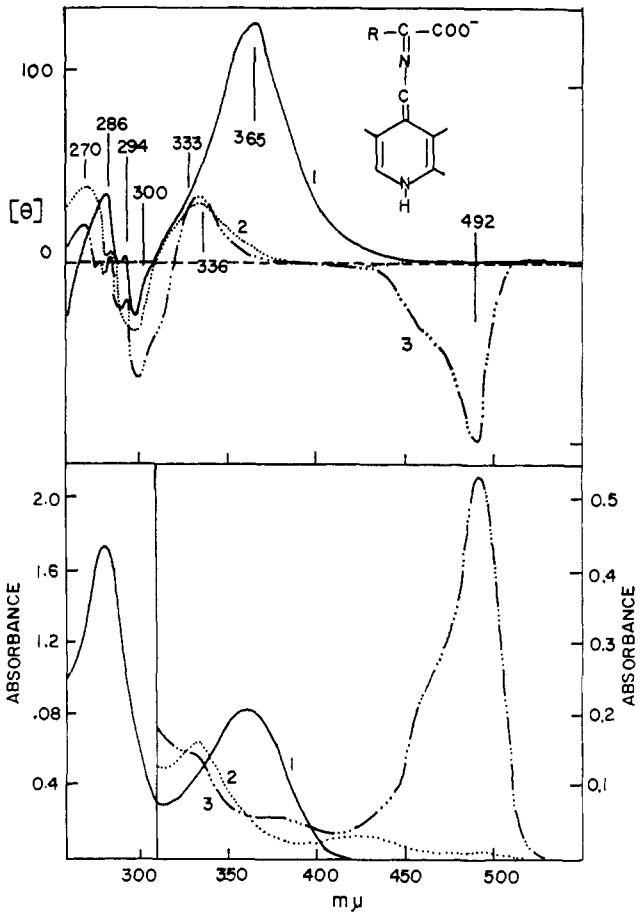


FIGURE 4: Circular dichroism (top) and absorptions spectra, (bottom) of the supernatant isozyme. (1) Enzyme alone, (2) enzyme in 0.1 M DL-aspartate. (3) Enzyme in presence of 0.04 M *erythro*-β-hydroxyaspartate. Enzyme concentration 1.23 mg/ml and in 0.5 M sodium tetraborate buffer (pH 8.9). Insert: structure of the semiquinoid enzyme–substrate complex absorbing at 490–500 mμ as proposed by Jenkins (1964) for the supernatant isozyme and by Schirch and Slotter (1966) in model systems.

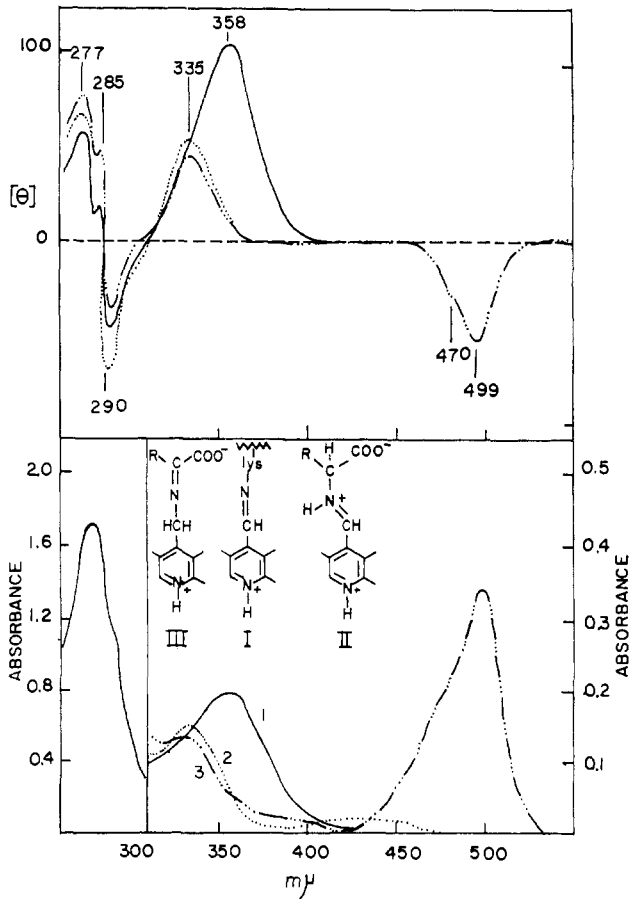


FIGURE 5: Circular dichroism (top) and absorption spectra (bottom) of the mitochondrial isozyme. (1) Enzyme alone. (2) Enzyme in the presence of 0.1 M DL-aspartate. (3) Enzyme in presence of 0.007 M *erythro*-β-hydroxyaspartate. Enzyme concentration 1.23 mg/ml and in 0.05 M sodium tetraborate buffer, pH 8.9. Insert: (I) pyridoxal enzyme at high pH, absorbance at 360 mμ. (II) Substrate–pyridoxal phosphate complex responsible for absorbance at 430 mμ. (III) Substrate–pyridoxal phosphate ketimine complex with absorbance at 330 mμ.

and a decrease in the positive amplitude at 284 mμ with the appearance of a trough at lower wavelength and a new positive band at 264 mμ lacking in substrate-free holoenzyme (Figure 3A).

erythro-β-Hydroxyaspartate is a substrate analog that transaminates very slowly with either isozyme. It is known to form an initial Schiff's base with the pyridoxal moiety of the transaminase and give a series of complexes absorbing at unique wavelengths. Of these complexes, the species absorbing at 490 mμ with a shoulder at 470 mμ have been characterized by Jenkins (1964) as a semiquinoid-type complexes of the pyridoxal phosphate in the enzyme with the amino acid lacking the α proton. The mitochondrial isozyme also shows this property but the absorption maxima are slightly shifted to 498 and 475 mμ. Other species absorbing at 336 mμ are probably the transition state ketimine forms of the substrate–pyridoxal phosphate–glutamate aspartate transaminase complexes (III in Figures 5 and 6). Our circular dichroism measurements in this visible region agree with the previous report using optical rotatory dispersion (Breusov *et al.*, 1964), of a Cotton effect centered about 500 mμ with a sign opposite to those given by free supernatant isozyme. As shown in Figures 5 and 6, both isozymes depict negative circular dichroism bands in the 490–500-mμ region and a characteristic shoulder at 470 mμ. The amplitude of these bands when divided by the absorption of the corresponding wavelengths, gives the dissymmetry factor $\Delta\epsilon/\epsilon$, which is different for each isozyme (Table III).

In recent circular dichroism measurements of the visible region of the supernatant isozyme in the presence of hydroxyaspartate (Ivanov *et al.*, 1967) reported a positive band at 360 mμ, we have been unable to confirm these findings but their substrate concentrations were too low to achieve saturation of the enzyme. Below 320 mμ, both isozymes show a series of transitions with dichroicity bands. The position and amplitude of the bands do not resemble those of free supernatant isozyme in the pyridoxal phosphate form, but those given by the pyridoxamine form of the supernatant isozyme with a large negative band at 300 mμ and positive bands centered at 294, 286, and 270 mμ (Figures 3 and 4). The circular dichroism patterns in this region of the mitochondrial isozyme are identical with those observed

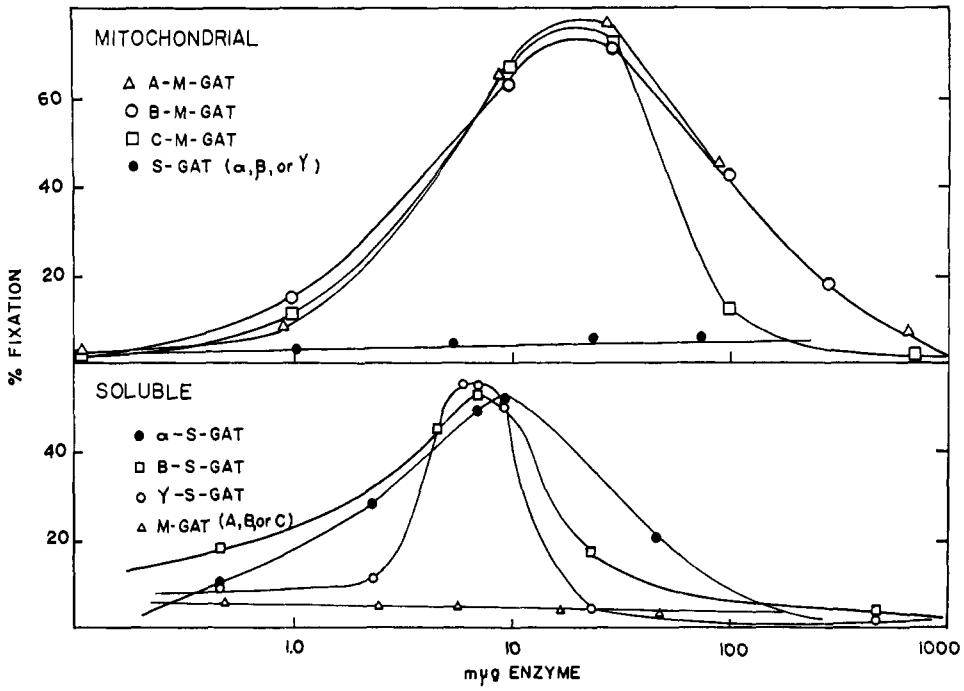


FIGURE 6: Complement fixation studies. Of anti C subform of the mitochondrial isozyme with the mitochondrial multiple forms at a serum dilution of 1:500 (top). Of anti α, supernatant isozyme (1:5000 serum dilution) with the supernatant multiple forms (bottom). Apoenzymes of any of the multiple forms fixed as much complement as their respective holoenzymes. Fixation of complement by either the supernatant or mitochondrial isozyme in the presence of 0.1 M L-aspartate or 0.04 M *erythro*-β-hydroxyaspartate was the same as in the absence of these substrates (see text).

in either the pyridoxal or pyridoxamine forms (Figures 3B and 5).

A spectrum of the supernatant isozyme saturated with L-aspartate at high pH displays maxima at 490, 430, and 333 mμ, indicative of the different equilibrium species of enzyme–substrate complexes present (Jenkins and Taylor, 1965). The circular dichroism spectrum in the visible region showed only a positive band in the 336-mμ region. In the aromatic region there is a negative band at 300 mμ and two positive bands at 286 and 270 mμ (Figure 4). Mitochondrial isozyme under identical conditions produces absorption bands at 330 and 430 mμ (Michuda and Martinez-Carrion, 1969b). The circular dichroism of aspartate-saturated mitochondrial isozyme is devoid of any band at 430 mμ and shows only a positive dichroic maximum at 330 mμ and a pattern in the aromatic region similar to that observed in the free enzyme (Figure 5). The dissymmetry factor, $\Delta\epsilon/\epsilon$, of the 330-mμ band differs for both isozymes (Table III). None of the isozymes changes its circular dichroism pattern in the far-ultraviolet region under conditions of substrate saturation. These circular dichroism patterns are identical with those in Figures 1 and 2 and were, therefore, omitted.

Microcomplement fixation methods are very sensitive to conformational changes in proteins because the complement fixing capacity of immune systems depends to a large extent on the affinity of the serum antibodies for the individual determinants in the antigen.

Since changes in structure or conformation of the protein antigen may directly or indirectly alter the antigenic determinant in the protein, this would then be followed by a variation in the interaction of the antigen with its specific antibody. Using the complement fixation technique, alterations in structure of simple proteins have been detected (Levine, 1962; Reichlin *et al.*, 1964; von Fellenberg and Levine, 1967). Similarly, conformational changes of multichained proteins like hemoglobin and lactic dehydrogenase have also been studied by this technique (Reichlin *et al.*, 1965; Cahn *et al.*, 1962). The effects of ligands in the conformation of the enzyme aspartate transcarbamylase have been reported by von Fellenberg *et al.* (1968). This method, therefore, provides an additional tool to probe the conformational relationship of the isozyme groups to each other, those of the multiple forms within each group, and the effect of substrates and coenzyme in protein conformation.

The results in Figure 6 clearly indicate no differences in the amounts or equivalent zones of complement fixed among the subforms within each group, eliminating, at least by this method, the possibility of conformational differences between the antigenic sites of the subforms in each isozyme. Since there were also no differences in the complement fixation curves between apo- and holoenzymes for the supernatant or mitochondrial isozyme, we can independently confirm our circular dichroism data which fails to detect conformational differences after removal of pyridoxal phosphate. When the complement fixations were run in the presence of L-aspartate (0.025 M) or *erythro*-β-hydroxyaspartate, (0.04 M) no differences were observed in the fixation curves of each isozyme.[1] Both isozyme groups failed to react immu-

[1] Experiments were run with D-amino acids as the proper controls because they do not bind to the enzymes.

TABLE III: Comparison of the Visible and Ultraviolet Circular Dichroic Bands for the Pyridoxamine Form and the Enzyme–Substrate Complexes.

Enzyme	Substrate	λ_{max}	$\Delta\epsilon/\epsilon^a \times 10^4$	$[\theta']^b$
Supernatant	Aspartate	430		
		365		
		221		−9,760
		336	5.87	
	α-Methylaspartate	430		
		366	13.9	
		221		−9,760
	Hydroxyaspartate	492	−5.78	
		430		
		360		
		336	7.15	
		221		−9,760
	Pyridoxamine form	333	12.5	
		232		
		221		−9,760
Mitochondrial	Aspartate	430		
		368		
		335	11.2	
		219		−12,000
	α-Methylaspartate	430		
		368	8.4	
		336		
		219		−12,000
	Hydroxyaspartate	494	−4.84	
		335	10.5	
		219		−12,000
	Pyridoxamine form	334	10.5	
		219		−12,000

[a] $\Delta\epsilon$ calculated for θ values and dividing by the absorbance. [b] In mean residue weight 114 and corrected for refractive index of the solvent.

TABLE IV: Helical Content of the Supernatant and Mitochondrial Isozymes by Comparison of the Circular Dichroic Spectra in the Ultraviolet with Those of Polypeptides of Known Conformation.

Enzyme	λ_{min}	% α Helix	Cross-over Point	Random Coil	β Configuration
Supernatant	207	37[a]	200	63	Little
Mitochondrial	207	40[a]	200	60	Little

[a] Using the method of Greenfield and Fasman (1969).

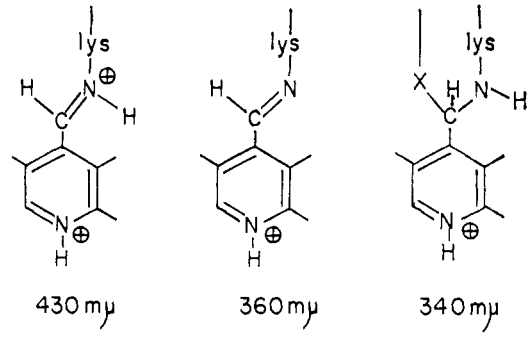
nologically with serum prepared against any of the multiple forms of the other isozyme, as it can be expected of two different molecular entities (Martinez-Carrion and Tiemeier, 1967).

Discussion

Perhaps the most striking general observation in the transaminases under study is that the mitochondrial and supernatant isozymes, structurally dissimilar proteins, have so many conformation features in common.

Circular dichroism spectra of helical polymers possess electronic transitions that can be fitted in three bands of varying intensities centered at 222, 206, and about 190 mμ. They are the contributions of the peptide group transitions $n\sim\pi^*$, $\pi-\pi^*$ (parallel polarized), and $\pi-\pi^*$ (perpendicularly polarized) (Holzwarth and Doty, 1965). The β conformation is also capable of producing dichroicity bands centered at 217 and 195 mμ with a crossover at 207 mμ (Townsend *et al.*, 1966). Recently, Greenfield and Fasman (1969) have developed a new procedure to interpret the contributions of the α helix, β configuration, and random coil in measured circular dichroism spectra. We have used this new method to estimate the type of structure that predominates in the glutamate aspartate transaminase isozymes. Both isozymes contain little β conformation but as much as 40% helix (Table IV). These values are, nevertheless, as absolute as the assumptions implicit in Greenfield and Fasman's work prove to be.

Both isozyme groups show identical dissymmetry factors in the 360–430-mμ region for the binding of pyridoxal phosphate. The multiple forms of the supernatant isozyme have a decrease in amplitude that corresponds to the appropriate amounts of "active" pyridoxal phosphate bound, with the α form being the highest and the low catalytic activity γ form the lowest. The low dichroicity of the 340-mμ region of the β and γ forms agrees with our previous assumption that the absorbing species at 340 mμ represents pyridoxal phosphate bound in forms other than the internal Schiff's base (Martinez-Carrion *et al.*, 1967). Contrasting the double-bond character of the Schiff's base characteristic of activity-bound pyridoxal phosphate, it might correspond to a tetrahedral carbon attached to group X in the protein. The same



interpretation should explain the absorbing species of the A, B, and C forms of the mitochondrial isozyme.

Since the mitochondrial and supernatant isozymes failed to cross-react immunologically it is unlikely that both possess identical tertiary structure. More significance might be attached to spectra in the visible region due to bound coenzyme. Secondly, since no differences in dichroicity can be detected in the ultraviolet region, the glutamate

aspartate transaminase multiple forms are apparently not "conformers" such as those by Kitto *et al.* (1960) for malic dehydrogenase.

Because the ultraviolet circular dichroism patterns are identical, any differences between apo- and holoenzymes must be finely localized in the protein or conformationally compensated and hence optically undetectable. The complement fixation data eliminates the latter possibility because an internal compensation of antigenic sites seems unlikely. Hence, the previously reported differences in the optical rotatory dispersion patterns of apo and holo supernatant isozyme (Fasella and Hammes, 1964) were probably measurements on enzyme preparations that were partially denatured in the process of preparing the apoenzyme.

The multiplicity of bands in the aromatic region of spectra given by the isozymes and their multiple forms seems to be diagnostic for environmental differences of some aromatic residue. In this case, the patterns given by each isozyme group are different and contrast their similarity in dichroicity pattern in the far-ultraviolet region. The reversal of the sign and variation of the center of the aromatic chromophore bands after binding of pyridoxal phosphate is a new observation. Pyridoxal phosphate itself when in solution or bound to other unspecific proteins such as bovine serum albumin (Johnson and Graves, 1966) has never been reported to show Cotton effects or dichroicity bands in this reuion. Even though studies with polypeptide models with aromatic residues have been carried out, it would be unwise to assign each band to a particular type of chromophore on each protein. Spectral shifts, overlapping bands, and energy level splitting in various protein environments would have to be known before making any reliable assignment. Myer (1968) also reported inversion of the dichroicity bands in the aromatic region of cytochrome *c* upon changes in oxidation states. These changes never showed significant alteration s in the ellipticity values even though there is overwhelming chemical and physical evidence for distinct conformational states for oxidized and reduced cytochrome *c*. The environment of these aromatic chromophores is provided largely by tertiary structure which is particularly susceptible to denaturing agents such as guanidine hydrochloride. The lower amplitude of the 289-mμ peak in the supernatant isozyme with increasing pH is interesting.

During the catalysis, both the absorption and optical properties are affected by changes in the environment of this chromophore.

Alterations in the dissymmetry of the transitions of the bound pyridoxal phosphate by inhibitor complexes, such as those formed with *erythro*-β-hydroxyaspartate, and those induced in aromatic chromophores adjacent to pyridoxal phosphate during reconstitution of the apoenzyme are complex. Stryer and Blout (1961), while working on the multiple binding of dye molecules to polyglutamic acids of known configuration, differentiated "configurationally induced" optical rotatory power, resulting from interaction of the dye with the environment of the asymmetric carbon, from "conformationally induced" power, resulting from the mutual interaction of dye molecules which have been ordered by the polymer helix. Since one molecule of pyridoxal phosphate or inhibitor is bound per molecule of transaminase subunit, the changes in the active transitions would fit the former category. Variations in the rotatory strengths of the bound pyridoxal phosphate in the presence of substrates could be due to changes in the coupling of transitions of pyridoxal phosphate with the protein during pyridoxal phosphate–substrate complex formation or in electrostatic perturbations produced by charged groups or dipoles of the protein.

Perturbations in the same aromatic region in the holoenzyme of the supernatant isozyme after binding of the substrate(s), may just be indicative of the environmental changes undergone by the aromatic chromophores of pyridoxal phosphate itself. They are less distinct in mitochondrial isozyme which may reflect an isozyme variance in the topology around those aromatic amino acids.

Enzyme–substrate complexes of supernatant isozyme absorbing in regions other than those shown by free enzyme may or may not be devoid of optical activity (Braunstein, 1964). In both isozyme groups, the internal aldimine species absorbing at 430 mμ at saturation levels of aspartate or the analog α-methylaspartate lacks a dichroic band indicating a shift in environment from that of the species absorbing at the same wavelength in the free enzyme. In addition, with α-methylaspartate there are species absorbing at about 360 mμ. They possess dichroic character but have a dissymmetry factor lower than that found with the free isozymes. The same observation applies to the species absorbing at 336 mμ at saturation levels of aspartate. Absorbances in the latter region should be due to the ketimine rather than the aldimine or pyridoxamine species. Qualitatively, credence is given to similar mechanisms of transamination for both isozyme groups to behave similarly in the formation of enzyme substrate complexes and their subsequent alterations. The quantitative comparison is represented in Tables III and IV *via* dissymmetry factors of these complexes. The minor differences most likely reflect subtle variations at the active sites of the supernatant and mitochondrial isozymes.

erythro-Hydroxyaspartate forms equilibrium mixtures of the ketimine (330 mμ) and a semiquinoid type of enzyme–substrate complex (490 mμ) (Jenkins and D'Ari, 1966b). The ketimine species shows a positive dichroic band like those given by the natural substrate aspartate. On the other hand, the semiquinoid type of intermediate shows a negative band with a low dissymmetry factor. This complex, because of its highly conjugated character, is necessarily planar (III in Figure 4). This implies an interconversion of a tetrahedral α carbon in the initial amino acid–pyridoxal phosphate complex (II in Figure 5) to a complex species coplanar with the pyridine ring of the coenzyme. The resulting conformational change probably modifies the substrate's interactions (over and above the amino group linkage) with the protein, leading to large alterations in the environment of the covalently linked amino acid–enzyme complex. This phenomenon would occur with both isozymes.

Conformational variation of protein amino acids at the active site would be a sufficient, though not necessary, condition for the changes observed. It should be recalled that the substrate, while covalently bound to the reporter chromophore is altered conformationally. These transformations in the coenzyme–substrate complex could distort its environment. There is no evidence on the number and nature of the enzyme–pyridoxal phosphate ligands. Changes in the conformation of the pyridoxal phosphate–substrate complex, although they could involve minor displacements of the

pyridine ring, do not necessarily imply rotation of the pyridoxal phosphate moiety about any single axis. Thus, postulations of such rotations on the basis of circular dichroism evidence (Karpeisky and Ivanov, 1966; Ivanov and Karpeisky, 1969) should be regarded as one of many hypothetical schemes. Since the substrate-induced localized changes do not vary the complement fixation properties of the isozymes, they must occur in a region relatively independent of the antigenic sites.

The presence of dichroicity in the pyridoxamine form of each isozyme group can be explained by a stiffening of the coenzyme environment due, perhaps, to hydrogen bonding between the amino group of the coenzyme and the ϵ-amino group of the active-site lysine (Fasella *et al.*, 1966).

Since the supernatant and mitochondrial isozymes show no similarity in the amino acid sequence of isolated peptides containing bound pyridoxal phosphate to the ϵ-amino group of a lysine residue (Morino and Watanabe, 1969) both enzymes must share similar features in the steric vicinity of the pyridoxal phosphate prosthetic group rather than in their primary structures. Credibility is given to these active-site topological similarities between the isozymes by: (a) circular dichroism data of the free enzymes or of the enzymes in the presence of substrates or their analogs; (b) proposed catalytic mechanisms for the isozymes; (c) the substrate affinities of the supernatant and mitochondrial isozymes (Michuda and Martinez-Carrion, 1969b); and (d) the change in sign of the aromatic region in the circular dichroism with resolution of the enzyme.

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