Mitochondrial Glutamate Aspartate Transaminase. Differential Action of Thiol Reagents with the Supernatant Enzyme*

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ABSTRACT: A comparative study of the sulfhydryl groups of mitochondrial and supernatant glutamic aspartic transaminase (GAT) is made to determine if they bear the same structure to function relationship in these two isozymes which are similar in catalytic purpose but distinct in their amino acid sequence and cytological localization. All ten of the sulfhydryl groups of the mitochondrial enzyme (M-GAT) react with *p*-mercuribenzoate (MB) but only six of ten in the apo and holo supernatant (S-GAT) isozyme do so. In the presence of urea all the sulfhydryl groups can be titrated with MB in each enzyme. The reaction with MB and the reversibility by mercaptoethanol of the MB induced inactivation are more rapid in M-GAT, indicating greater accessibility of sulfhydryl groups. When bound with MB, most of the structure is maintained in holo S-GAT but not in apo S-GAT or holo and apo M-GAT as evidenced by circular dichroism, complement fixation, and sedimentation velocity data. After treatment with MB, pyridoxal phosphate binding is not disturbed in either isozyme but pyridoxamine phosphate dissociates more rapidly from the M-GAT than from S-GAT. The p*K* of the enzyme-bound pyridoxal phosphate chromophore is increased in S-GAT when the sulfhydryl groups are blocked with MB. This is not true of M-GAT. The inhibition of binding of the pseudo substrates, *erythro*-β-hydroxyaspartate and α-methylaspartate, by S-GAT enzyme follows its inactivation as a function of sulfhydryl groups bound with MB. A similar effect does not occur in the M-GAT. Maleate, bromopyruvate, and iodoacetamide combine with only four thiol groups in both isozymes when in the native state. However, after 24 hr in 8 M urea, iodoacetamide can react with ten thiol groups of M-GAT and six thiols of S-GAT. In M-GAT catalytic activity and substrate affinity are maintained if all ten of the sulfhydryl groups are carboxyamidomethylated. This contrasts with the great inhibition of catalysis and impairment of substrate binding in S-GAT after more than four sulfhydryl groups are blocked with either iodoacetamide or MB. It is concluded that M-GAT does not possess sulfhydryl groups essential for catalysis. Indirect evidence, however, seems to indicate that in S-GAT the thiol groups may be critical to activity because of their steric proximity to the active center.

Glutamic aspartic transaminase (GAT)[1] (EC 2.6.1.1) exists as two cytologically distinct isozymes, the mitochondrial (M-GAT) and supernatant (cytosol) (S-GAT) forms. Although the two enzymes catalyze the same reaction, they differ markedly in primary structure and chemical and physical properties (Martinez-Carrion and Tiemeier, 1967; Michuda and Martinez-Carrion, 1969). Because the two proteins are localized in different cytological compartments of the same tissue, it is important to know the structural features they share as well as the amino acids at their active site(s). This knowledge may lead to an explanation of the chemical basis for the catalysis of the same reaction by two such dissimilar proteins with identical specificity but with subtly different catalytic steps (Michuda and Martinez-Carrion, 1969, 1970; Boyde, 1968). Thus, the amino acid residues that might affect the active-site chromophore environment, directly or indirectly, are of special interest. So far only one small portion of this environment, the amino acid sequence of the peptides containing bound pyridoxal phosphate to the ε-amino group of a lysyl residue, is known to be different in both enzymes (Morino and Watanabe, 1969). In addition, the spectroscopic characteristics of the enzyme–substrate complexes indicate that the isozymes share similar features in the steric vicinity of the pyridoxal phosphate prosthetic group (Martinez-Carrion *et al.*, 1970b).

In this work we have chosen to follow the effects of sulfhydryl group inhibitors because the catalytic activity of one isozyme, S-AGT, is sensitive to MB (Polyanovsky, 1962; Turano *et al.*, 1963). In other aminotransferases, there is uniform behavior with respect to the blocking of thiol groups. L-Alanine (Matsuzawa and Segal, 1968), glutamine (Hsu, 1960), tyrosine (Kenny, 1959), and ornithine (Vogle and Kapac, 1960) transaminases are all inactivated by this treatment but D-alanine transaminase (Martinez-Carrion and Jenkins, 1965) is not. Therefore, the possible participation of a thiol group in all enzymatic transaminations remains questionable.

The purpose of this work is to ascertain whether the thiol groups participate in enzymatic transamination and whether they have the same structure to function relationship in the two isozymes.

Experimental Section

Chemicals. Pyridoxal phosphate, iodoacetamide, mercaptoethanol, and cysteinesulfinic and α-methylaspartic acids were purchased from Sigma Chemical Co. MB was purchased from Mann Research Laboratories. Dithiothreitol and *erythro*-β-hydroxyaspartic acid were obtained from Calbiochem.

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[1] Abbreviations used that are not listed in *Biochemistry 5*, 1445 (1966): GAT, glutamic aspartic transaminase; S-GAT, supernatant glutamic aspartic transaminase; M-GAT, mitochondrial glutamic aspartic transaminase; MB, *p*-mercuribenzoate.

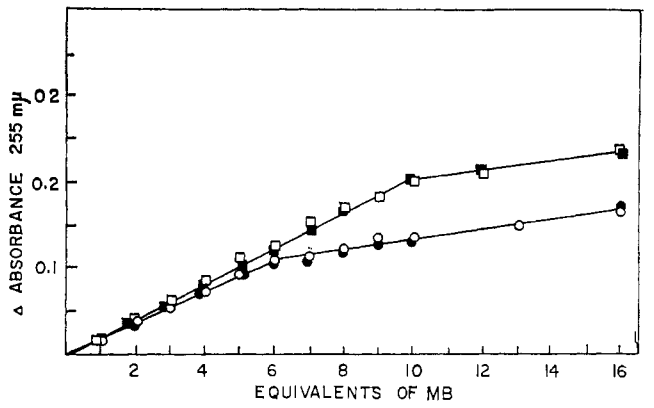

FIGURE 1: Titration of glutamic aspartic transaminase with MB. Enzyme concentration of 1.95×10^{-6} M titrated with aliquots of 2.54×10^{-3} M MB in 0.1 M sodium acetate buffer pH 5.0 at 4°. (●) Holo S-GAT; (○) apo S-GAT; (■) holo M-GAT; (□) apo M-GAT.

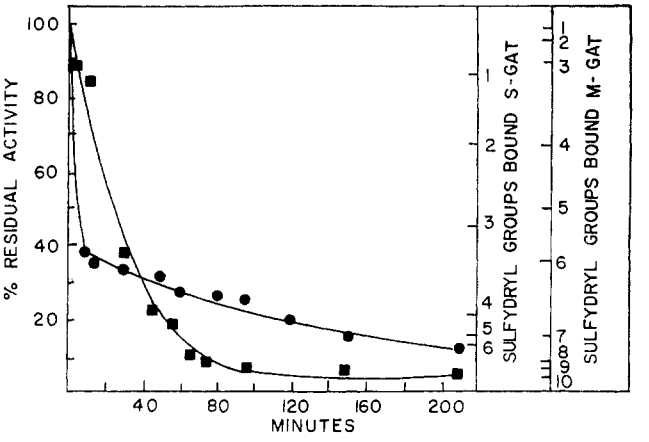

FIGURE 3: Inactivation of aspartate transaminase by MB. S-GAT concentration, 0.375×10^{-5} M; M-GAT concentration, 0.86×10^{-5} M; reacted with a 30-fold molar excess of MB in 0.1 M sodium acetate buffer (pH 5.3) at 4°. (●) Holo S-GAT; (■) holo M-GAT.

Enzyme Preparation. M- and S-GAT were prepared from pig hearts as previously described (Martinez-Carrion *et al.*, 1967; Michuda and Martinez-Carrion, 1969). Preparation of the apo form of S-GAT was accomplished by the method of Martinez-Carrion *et al.* (1970a). Protein concentrations were calculated from optical densities at 280 mμ where ϵ is 140,000 for a molecular weight of 94,000 (Feliss and Martinez-Carrion, 1970).

Spectrophotometric Studies. Absorption spectra were recorded with a Cary Model 15 recording spectrophotometer equipped with a 0–0.1 absorbance unit expanded-scale slidewire. Spectrophotometric titrations were done on a Gilford 2000 recording spectrophotometer with zero offset attachment and an automatic blank compensator. The pH values were determined by a Sargeant Model DR pH meter equipped with a Corning glass combination electrode accurate to ±0.01 pH unit at 25°.

Circular Dichroism. These measurements were made with a Cary Model 60 spectropolarimeter equipped with a circular dichroism attachment. Silica cells with a light path ranging from 1 to 10 mm were used. The θ values were recorded directly from the instrument. Molecular ellipticity, $[\theta]$, was calculated from 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/cm³. $[\theta']$ refers to the molecular ellipticity values where the refractive index corrections have been included.

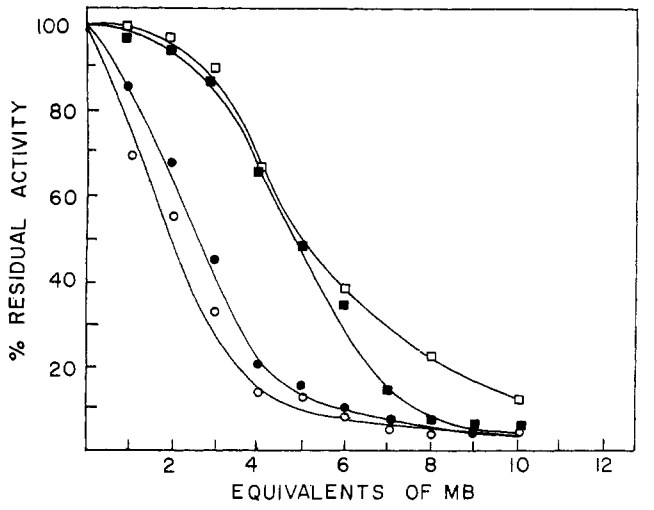

FIGURE 2: Residual activity of the isozymes bound with MB. Enzyme concentration, 2.15×10^{-6} M, reacted with aliquots of 2.38×10^{-3} M MB in 0.1 M sodium acetate buffer (pH 5.3) for 3 hr at 4°. 100% is activity of enzyme without MB added. (●) Holo S-GAT; (○) apo S-GAT; (■) holo M-GAT; (□) apo M-GAT.

Sulfhydryl Reagents. MB titrations employed the method of Boyer (1954). Titrations were done by adding a different number of equivalents of MB to a series of equivalent enzyme samples; the reaction was allowed to proceed for 12 hr. Similar results were obtained with different enzyme preparations. Maleated enzyme was prepared by heating the enzyme for 3 min at 60° in 0.08 M maleate (pH 6.0). Excess maleate was removed by passage through a Sephadex G-25 column. Reactions with 0.1 M bromopyruvate were in 0.5 M phosphate buffer (pH 6.0) and incubated for 2 hr at 37°. The enzymes were then precipitated with 90% ammonium sulfate and dialyzed against 0.1 M phosphate buffer (pH 6.0). Reaction with 0.4 M iodoacetamide was in 0.1 M potassium phosphate buffer (pH 6) for 24 hr at 23° or, under the same conditions in 8 M urea, after having kept the enzymes for 24 hr at 23° in an 8 M urea solution that had been deionized by passage through a mixed-bed resin. At the end of the reaction the urea was always removed by dialysis against distilled water. Control samples were subjected to identical treatment in the absence of the thiol reagent. After these treatments the enzymes were titrated with MB to ascertain the sulfhydryl groups bound.

Enzyme Activity. The enzyme activities of the holoenzymes were determined by the method of Lis (1958) or Michuda and Martinez-Carrion (1970). The apoenzymes were assayed by the same method after preincubation of the samples with 10^{-4} M pyridoxal phosphate in 0.1 M Tris-chloride buffer (pH 8.6) for 15 min at room temperature.

Ultracentrifugation. The enzyme solutions were in 0.1 M sodium acetate buffer (pH 5.3) at 3 and 4 mg per ml. The sedimentation velocities of proteins were determined with a Spinco Model E analytical ultracentrifuge equipped with a phase plate as a schlieren diaphragm. The rotor was run for 1 hr at 11.2° at 56,000 rpm. Double-sectored cells with a dis-

TABLE I: [θ'] Dichroicity at 220 mμ as a Function of Bound MB.[a]

Moles of MB/Moles of Enzyme	Apo S	Holo M	Apo M
0	−9760	−11,900	−11,900
2	−9760	−11,900	−11,900
4	−8920	−11,200	
6	−7820	−10,700	−7,920
8		−10,000	−7,370
10	−7050		−6,000

[a] Enzyme concentration, 8.96×10^{-7} M, reacted with aliquots of 7.22×10^{-4} M MB for 3 hr in 0.1 M sodium acetate buffer at 4°, pH 5.0. Spectra taken in 1-mm silica cells. Holo S-GAT showed no apparent change.

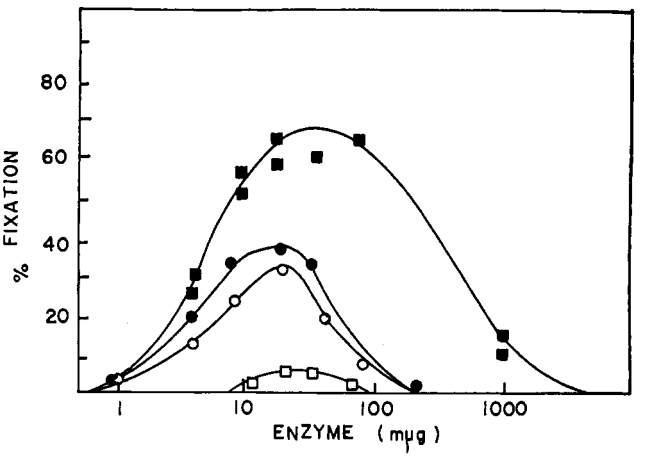

FIGURE 4: Complement fixation curves for S-GAT: (●) native, (○) with 6 equiv of MB bound. M-GAT: (■) native, (□) with 10 equiv of MB bound.

placed meniscus were used for direct comparison of the rate of migration of native and MB-bound enzyme.

Immunochemistry. The complement fixation studies were performed by using the microcomplement technique of Wasserman and Levine (1961).

Amino Acid Analyses. Acid hydrolysates were prepared as described by Moore and Stein (1963) and the analyses performed in a Technicon Autoanalyzer.

Results

Sulfhydryl Group Binding with Mercuribenzoate. All ten of the sulfhydryl groups in apo and holo M-GAT can be titrated with MB. However, only six of the ten in the supernatant enzyme are available for reaction (Figure 1). No additional sulfhydryls are exposed in the apoenzyme of either isozyme, indicating no masking of a sulfhydryl groups in cofactor binding. All ten sulfhydryl groups can be titrated in 8 M urea in both holoenzymes. The inactivation as a function of sulfhydryl groups bound is much pronounced for S-GAT than for M-GAT (Figure 2). Blocking of the first three sulfhydryl groups of M-GAT appears to have almost no affect on activity. The apoenzyme's inactivation follows closely their holo forms.

Aliquots of a reaction mixture containing enzyme at 4° and a 30-fold excess of MB were taken at various times and assayed for activity. When these activity losses are correlated with equivalents of MB bound (Figure 2), a behavior of inactivation paralleling sulfhydryl groups bound can be plotted as in Figure 3. All ten of M-GAT's sulfhydryl groups are bound within 100 min; however, a much longer reaction time is needed to inactivate and bind only six of the ten thiol groups in the supernatant enzyme.

Structural Studies. To ascertain whether the loss of activity produced with binding sulfhydryl groups with MB could be explained by large losses of structure, circular dichroism spectra were taken for isozymes bound with various ratios of MB. The 220-mμ dichroic minimum was used as an index of the enzyme's average total of secondary and tertiary structure. According to the method of Greenfield and Fasman (1969), native S-GAT would have 37% helical content while native M-GAT has 40% (Martinez-Carrion *et al.*, 1970b). Table I shows the loss of [θ'] as a function of bound sulfhydryl groups. By this criterion alone, holo S-GAT appears to remain intact but apo S-GAT loses about 20% of its structure with six sulfhydryl groups bound. Holo M-GAT loses about 15% of its structure after all sulfhydryl groups are bound while approximately 50% is lost in the apoenzyme.

After reacting with bromopyruvate or iodoacetamide, in the absence of urea, there is full retention of structure. The lack of major conformational changes by holo S-GAT with six sulfhydryl groups bound with MB is further evidenced by the microcomplement fixation data. On the other hand, there is loss of structure when all accessible (ten) sulfhydryl groups of M-GAT are bound with MB (Figure 4).

The sedimentation coefficients, $s_{20,w}$, of S-GAT (5.6 S) and M-GAT (5.6 S) remained essentially unaltered after reacting with excess MB. Thus, the loss of activity after binding MB does not seem to be due to dissociation into subunits.

The complete reversibility of MB inactivation for both isoenzymes by mercaptoethanol is illustrated in Figure 5. In this reversal, as also for the total times of inactivation (Figure 3), M-GAT regains 90% of its activity faster than S-GAT. Re-

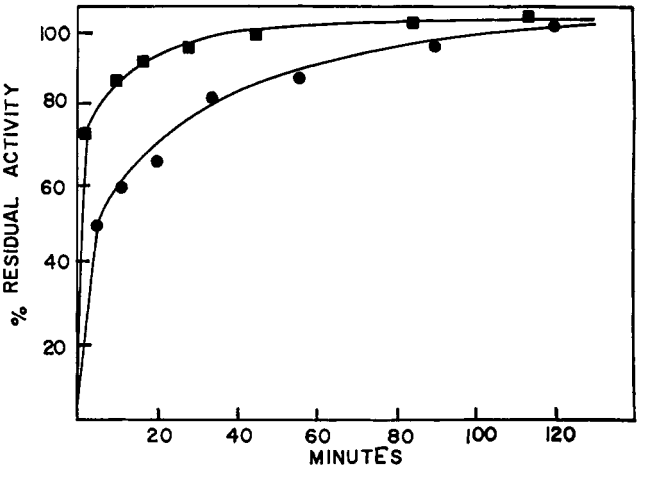

FIGURE 5: Reversal of MB inactivation of enzyme by mercaptoethanol. Enzyme concentration, M-GAT, 0.86×10^{-5} M (■); S-GAT, 0.735×10^{-5} M (●); reacted with a 30-fold molar excess of MB for 3 hr at 4° in 0.1 M sodium acetate buffer (pH 5.3). Reversed by adding 20 μl of mercaptoethanol directly into 1 ml of enzyme.

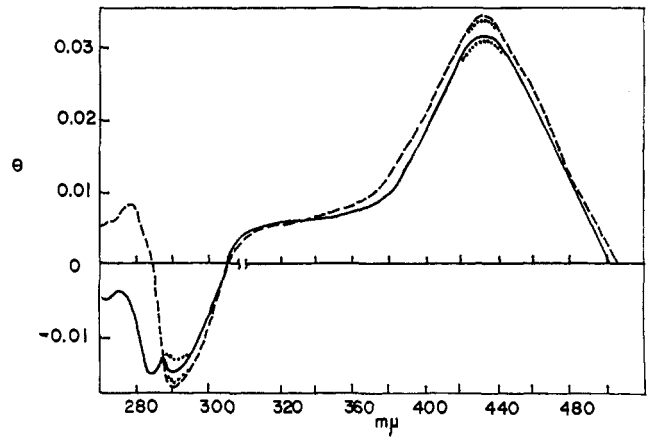

FIGURE 6: Circular dichroism spectra of aspartate transaminase. Enzyme concentration, 4.07 × 10^{-5} M reacted with a 10-fold molar excess of MB, in 0.1 M sodium acetate buffer (pH 5.33) for 3 hr. (——) Native holo S-GAT; (----) native holo M-GAT; (· · · ·) both isozymes bound with MB.

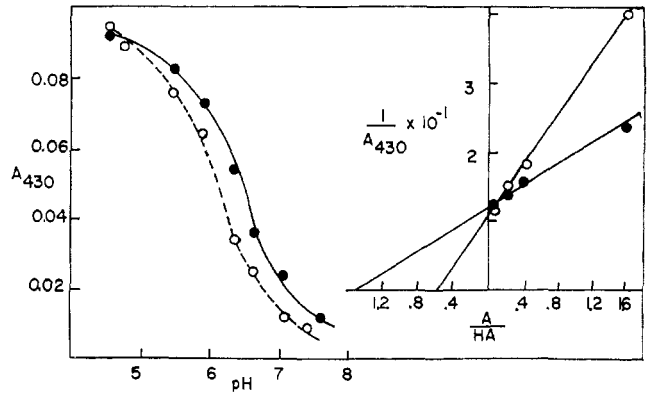

FIGURE 8: Acid dissociation constant of the chromophore of S-GAT bound with MB. Enzyme was reacted with 10-fold molar excess of MB. The enzyme was then adjusted to the indicated pH with cacodylate buffer so the final concentration of buffer was 0.1 M and of enzyme 1.205 × 10^{-5} M. (●) Enzyme fully bound with MB; (○) native enzyme.

versal of inactivation by 0.001 M dithiothreitol yielded similar, but slower, patterns of behavior for the two isozymes. After 44 hr M-GAT regained 95% of its activity while S-GAT regained only 55%.

Coenzyme Binding. The holoaspartate transaminases have positive optically active transitions in the 430-mμ region at low pH due to chiraly bound pyridoxal phosphate (Martinez-Carrion *et al.*, 1970b; Breusov *et al.*, 1964). One negative dichroic band centered at 290 mμ, which is presumably due to the interaction of pyridoxal phosphate with aromatic residues, becomes positive with changes in cofactor binding or environment. This may be caused by changes in pH or apoenzyme formation (Martinez-Carrion *et al.*, 1970b). These two wavelengths, 430 and 290 mμ, therefore, can be used as indices of changes in cofactor binding. Examination of the 430-mμ maximum and 290-mμ minimum shows no apparent change after binding all reactive sulfhydryl groups in the isozymes with MB (Figure 6). On the other hand, it has been postulated that the binding of sulfhydryl groups by MB enhances the release of pyridoxamine phosphate in S-GAT (Turano *et al.*, 1964). Questioning whether the same affect is operative in M-GAT, a comparison was made on the rates of release of pyridoxamine phosphate from the isozymes in which all sulfhydryl groups were previously bound with MB. Since the pyridoxamine enzyme exhibits a positive optically active maximum at 330 mμ due to chiraly bound pyridoxamine phosphate, the release of the cofactor can be monitored by the decreases of the 330-mμ maximum as apoenzyme is formed as a function of time. Figure 7 shows the time-dependent release of pyridoxamine phosphate from the isozymes when all accessible sulfhydryl groups are bound with MB. The more rapid release of pyridoxamine phosphate by the M-GAT is evident. The first-order rate constants of the release of pyridoxamine phosphate were 0.945 hr^{-1} for M-GAT and 0.192 hr^{-1} for S-GAT.

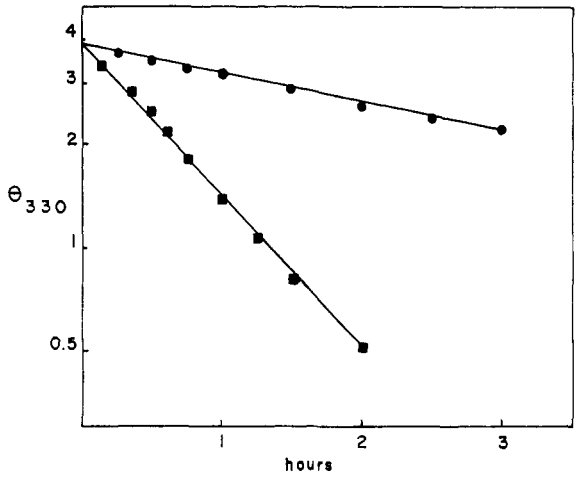

FIGURE 7: First-order plot of the release of pyridoxamine phosphate by enzyme bound with MB at 2°. Enzyme concentration, 4.7 × 10^{-5} M, reacted with a 20-fold molar excess of MB, for 18 hr in 0.1 M sodium acetate buffer (pH 5.0) at 2°. θ_{330} values refer to the direct dichroicity measurements at 330 mμ where full-scale chart deflection corresponds to 0.04 deg. Aliquots of cysteinesulfinic acid, 10 μmoles/ml (pH 6.0), were added to form pyridoxamine phosphate. (●) 330, S-GAT; (■) 330, M-GAT.

Influence of Binding of Sulfhydryl Groups with MB on the pK of the Enzyme-Bound Pyridoxal Phosphate. The holo transaminases are known to be pH sensitive, changing from yellow (absorbance at 430 mμ) at low pH to colorless (absorbance at 360 mμ) at high pH. The shift with pH is characterized by a sharp isosbestic point, suggesting that the color is produced by the addition of a proton to the enzyme chromophore, pyridoxal phosphate (Jenkins *et al.*, 1959). The pK of the dissociation of this proton in cacodylate buffer is 5.5 for both isozymes. Cadodylate buffer is chosen because it has a poor binding constant with the enzymes and thus has little effect on the apparent value of the pK by anion effects (Cheng *et al.*, 1971). Plotting $\Delta A_{430\,m\mu}$ *vs.* pH produces a sigmodial curve whose half-height should yield the pK of the bound pyridoxal phosphate. Since the isozymes are unstable at extreme pH values when bound with MB, the upper and lower limits of the curve are tenuous. Hence, a more accurate treatment of the data is employed. If $1/\Delta OD_{430}$ is plotted *vs.* A/HA, where A/HA is calculated from pH = pK − log (HA/A) taking the pK of the cacodylate buffer to be 6.2, the value of the pK of the bound pyridoxal phosphate can be calculated.

Figure 8 illustrates the sigmodial curve obtained from spectral data and the secondary plot used to calculate the pK of the chromophore. The pK of the bound pyridoxal phos-

TABLE II: Acid Dissociation Constants of the Chromophore.[a]

	pK of Chromophore	
	Enzyme	Enzyme with MB
Supernatant	5.50	6.35
Mitochondrial	5.50	5.50

[a] Details in Figure 8.

phate is unaffected by binding sulfhydryl groups in M-GAT but the binding of the groups in S-GAT increases the value of the pK by 0.8 unit compared to the native enzyme (Table II).

Reaction of Sulfhydryl Groups with Maleate, Bromopyruvate, and Iodoacetamide. In the absence of denaturing agents, the catalytic activity of both transaminases is unaffected after treatment with maleate, bromopyruvate, and iodoacetamide, even though four sulfhydryl groups are bound by these thiol reagents (Figure 9). After keeping the enzymes in 8 M urea for 24 hr, other sulfhydryl groups became accessible to iodoacetamide. As indicated in the figure, M-GAT retains 90–100% of its catalytic activity after carboxamidomethylation of all ten thiol groups. Similar treatment of S-GAT induces the loss of most of its activity following the blocking of the fifth and sixth sulfhydryl groups. The residual activity (10–15%), however, could not be eliminated by longer pretreatment with urea or increased molarity of iodoacetamide. Some properties of the isozymes treated with these reagents are shown in Table III. Clearly, carboxyamidomethylation of all sulfhydryl groups leads to a M-GAT preparation that is catalytically indistinguishable from the native enzyme. On the other hand, binding of more than four thiol groups leads to a substantial inactivation of S-GAT.

Substrate Binding. To measure the effect of MB-bound sulfhydryl groups on substrate binding, the substrate analog, *erythro*-β-hydroxy-L-aspartate, was used. The spectral absorption of the enzyme–substrate complex of this compound at alkaline pH exhibits a strong maximum at 490 mμ for the S-GAT and at 497 mμ for M-GAT. This maximum results from the semiquinoid form of the enzyme–substrate complex (Jenkins, 1964). If substrate binding is affected, this can be reflected in the decrease of the 490-mμ or 497-mμ maxima as a function of the number of sulfhydryl groups bound with MB. If 100% is taken as the height of the 490- or 497-mμ maxima with native enzyme, a correlation can be made of per cent binding as a function of peak height with number of bound sulfhydryl groups (Figure 10A). The inhibition of binding is much greater for S-GAT and closely follows its curve of inactivation as a function of bound sulfhydryl groups. However, both isozymes with only four thiol groups bound with bromopyruvate or iodoacetamide retain 90% activity and bind almost the same amount of substrate as the native enzyme. After all thiol groups are blocked with iodoacetamide, M-GAT still retains its substrate binding ability. In contrast, S-GAT loses its ability to bind hydroxyaspartate by the time its sixth sulfhydryl group has been carboxamidomethylated.

A change in the affinity of the substrate for the enzyme is reflected in a change in the dissociation constant of the enzyme–substrate complex. The K_d dissociation constant for the substrate can be calculated using the equation

TABLE III: Some Properties of the Aspartate Transaminases Treated with Maleate, Bromopyruvate, and Iodoacetamide.

Reagents and Condns for Modification[a]	Thiol[b] Content (moles/mole) S[c]	M[d]	Act. (%)	CM-Cys Content[e] (moles/mole)
None	6		100	0
		10	100	0
Maleate, 0.08 M;	2		100	
3 min, 60°		6	100	
Bromopyruvate, 0.1 M;	2		100	4.1
2 hr, 37°		6	100	3.9
Iodoacetamide, 0.4 M;	2		90	4.0
24 hr, 5°		6	100	3.8
Iodoacetamide, 0.4 M;	0.5		10	6.3
24 hr, 5°; 8 M urea		0	100	9.2

[a] Details of conditions given in text. [b] Determined by titration with MB. [c] S, supernatant aspartate transaminase. [d] M, mitochondrial aspartate transaminase. [e] From amino acid composition of acid hydrolysates.

$$\frac{1}{\Delta A_{490\ \mathrm{II}\ 497}} = \frac{K_d + (S)}{(\epsilon_2 - \epsilon_1)(E \cdot S)}$$

where E is the enzyme concentration, S is the concentration of substrate, and ϵ_1 and ϵ_2 are the extinction coefficients at 490 mμ of the enzyme and enzyme–substrate complex, respectively. By titrating a known concentration of enzyme with substrate and plotting $1/\Delta A_{490}$ *vs.* 1/(S), the value of the dissociation constant can be determined from the intercept on the abscissa

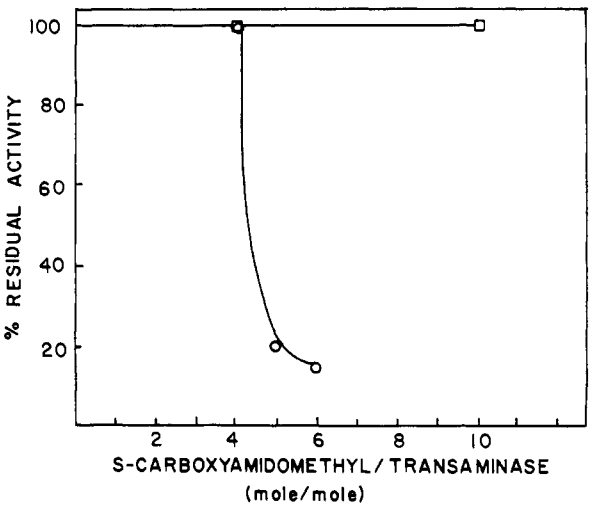

FIGURE 9: Residual activity of the isozymes after reaction of thiol groups with iodoacetamide. The points with four carboxyamidomethylated residues were obtained at enzyme concentrations of 2×10^{-5} M reacted with 0.4 M iodoacetamide in 0.1 M potassium phosphate buffer for 24 hr at 23°. The other points were obtained under the same experimental conditions but in 8 M urea and after the enzymes had been preincubated with the urea for 24 hr at 23°. The amount of free thiol groups after iodoacetamide treatment was measured by titration with MB after removal of urea and residual free iodoacetamide by dialysis. (○) S-GAT and (□) M-GAT.

TABLE IV: Dissociation Constants of the Isozymes.[a]

Substrates	K_d (mM)	
	Mitochondrial	Supernatant
erythro-β-Hydroxyaspartate		
Native	0.2	0.4
Enzyme with bromopyruvate or iodoacetamide	0.2	0.4
Enzyme with MB	1.25	1.66
α-Methylaspartate		
Native	2.5	3
Enzyme with MB	8	∞

[a] Details as in Figures 10B and 11. The dissociation constants were determined with ten thiols modified in M-GAT and six thiols modified in S-GAT.

(Figure 10B). The resultant values for K_d with both isozymes are reported in Table IV. It should be noted that the ordinate values of Figure 10B should be multiplied by ten for both isozymes for the MB–iodoacetamide line.

Formation of the 490-mμ absorption maximum depends not only on the presence of an initial enzyme–substrate complex but on the labilization of the α-hydrogen from the amino acid substrate. Therefore, it should be determined whether binding takes place independently of the formation of 490-mμ-absorbing species. To this end, circular dichroism changes were monitored. At high pH both M- and S-GAT possess a dichroic maximum at 360 mμ which disappears when *erythro*-β-hydroxyaspartate is added. By titrating a known concentration of the enzyme with this amino acid, a dissociation constant may be determined graphically from a plot of $1/\theta_{360}$ against 1/hydroxyaspartate, where θ_{360} is the amplitude at 360 mμ (Peterson and Martinez-Carrion, 1970). The plots are linear for both native and MB-bound enzymes as indicated in the legend in Figure 10. This approach gives results identical with those obtained by the monitoring of the 490-mμ absorbance. Therefore, when all accessible sulfhydryl groups have been blocked with MB in both isozymes, their affinity for substrate binding is impaired.

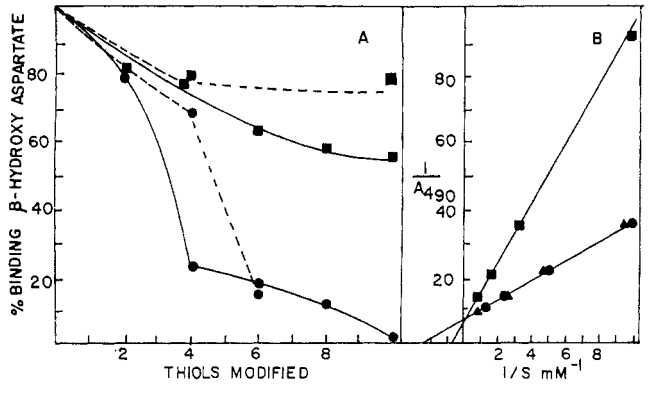

FIGURE 10: (A) Apparent binding of *erythro*-β-hydroxy-L-aspartate by enzyme with bound sulfhydryl groups. Enzyme concentration 0.604×10^{-5} M in 0.1 M sodium pyrophosphate buffer (pH 8.6) reacted with aliquots of 6.05×10^{-3} M MB or 0.1 M bromopyruvate. Final concentration of hydroxyaspartate, 40 mM. (●) Holo S-GAT; (■) holo M-GAT; (———) MB-modified enzyme; (———) bromopyruvate- or iododoacetamide-treated enzyme. 100% equals the absorbance at 490 or 497 mμ with native enzyme under similar conditions. (B) Spectrophotometric titration of S-GAT with β-hydroxyaspartate. (●) Native enzyme; (▲) enzyme with four sulfhydryl groups bound with bromopyruvate or iodoacetamide; (■) enzyme with six sulfhydryl groups bound with MB or iodoacetamide. Titrations were performed at 490 mμ with 0.945×10^{-5} M enzyme in 0.1 M sodium pyrophosphate buffer (pH 8.8). The ordinate values for the iodoacetamide–MB curve should be multiplied by a factor of ten. When changes in dichroicity ($\Delta\theta$) at 360 mμ were monitored and $\Delta\theta_{360}$ plotted *vs.* 1/hydroxyaspartate they gave identical results as reported for the absorbancy changes at 490 mμ.

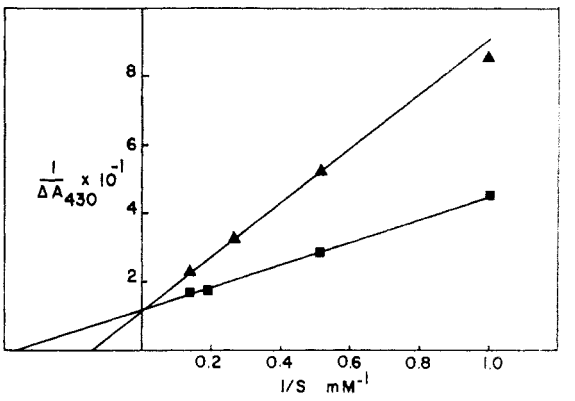

FIGURE 11: Spectrophotometric titration of M-GAT with α-methylaspartate. (■) Native enzyme; (▲) enzyme with ten sulfhydryl groups bound with MB. Titrations were performed at 430 mμ with 4.7×10^{-5} M enzyme at 2° in 0.1 M sodium pyrophosphate buffer (pH 8.1). Native S-GAT gave results similar to those of native M-GAT; however, under the same experimental conditions, no increase in absorbance at 430 mμ or a decrease of the original absorbance at 360 mμ could be detected upon addition of α-methyl aspartate of S-GAT with six thiols bound with MB.

Independent tests of the impairment of substrate affinity can be provided by measuring the binding of α-methylaspartate. With this compound only the initial covalent enzyme–substrate complexes are formed because subsequent catalytic events are not possible due to the presence of the α-methyl group. In this situation the absorbance at high pH with maximum at 360 mμ, upon addition of α-methylaspartate, gives rise to a new absorption maximum at 430 mμ due to the formation of an enzyme–substrate complex (Fasella *et al.*, 1966). It can easily be shown that by plotting 1/absorbance changes at 430 mμ against 1/α-methylaspartate concentration, the dissociation constant values can be calculated from the intercepts on the abscissa (Figure 11). Thus, binding of α-methylaspartate is greatly impaired subsequent to blocking of all accessible thiol group in either isozyme (Table IV).

Discussion

Although both isozymes contain ten sulfhydryl groups, in the absence of denaturing agents only six are available to MB in the supernatant isozyme as opposed to all ten in the mitochondrial form. Even with a 20% loss of structure in apo S-GAT, only six sulfhydryl groups can be titrated so it appears this isozyme has four deeply buried residues. These four groups which correspond to the very slow reacting groups described by Voratnitskaya *et al.* (1968) can only be titrated with MB in urea. The titration of all ten of the M-GAT sulfhydryl groups is probably a combination of residues initially more exposed and further availability of these thiols as the

enzyme structure loosens on binding MB. The more rapid reversal of MB inhibition of M-GAT by mercaptoethanol reinforces this concept.

Even though thiol reagents affect catalytic activity, a free sulfhydryl group is not essential for enzymatic transamination in M-GAT. This is illustrated by the full activity of the enzyme after all ten thiol groups had been carboxamidomethylated (Table III). That sulfhydryl groups are not essential for catalysis is less conclusive in S-GAT. Only the fact that partial residual activity is still available after all six accessible thiol residues have reacted with iodoacetamide would favor the concept of nonparticipation of these residues in catalysis.

It is of interest to note that there is no loss of activity or substrate binding in either isozyme after four sulfhydryl groups have been carboxamidomethylated. However, analogous treatment with MB produces substantial inactivation and substrate binding impairment in only S-GAT. This isozyme seems more sensitive to sulfhydryl group manipulations. Indeed, the great loss in catalysis and in substrate binding ability observed after blocking of the fifth and sixth thiol groups would indicate a great need for maintaining these two thiols free. These residues are not easily accessible since they can only be carboxamidomethylated under protein denaturing conditions and react very slowly with MB (Figure 3). The sluggishness of two thiols has also been noticed recently by Torchinsky and Sinitsina (1970).

The catalytic inactivation of the MB treated isozyme of GAT, as well as the inactivation of S-GAT after binding of all MB-accessible groups with iodoacetamide, could, in principle, be interpreted as losses of structure, size, or charge effects produced by the bound reagent, interference with cofactor binding, loss of ability to bind substrate, or a combination of all or some of the above.

The overall structure of the holo S-GAT seems to remain unaltered after MB treatment as shown by circular dichroism, complement fixation, and sedimentation velocity. Even though these techniques can miss subtle or localized conformation changes, they can as a whole be taken as indicative that inactivation cannot be explained by gross structural losses alone. These results are also in agreement with the observations of Torchinsky (1964) who found no difference in nagarse activity after MB treatment of holo S-GAT. On the other hand, apo S-GAT loses approximately 20% of its overall structure upon binding of MB; but blocking of the thiol groups with this reagent leads to losses of activity indistinguishable from those observed in holo S-GAT under identical treatment. Thus, the observable deterioration of structure in apo S-GAT cannot alone explain the diminished enzymatic activity. Since M-GAT when bound with MB loses structure in both the holo and apo forms, it is likely that the inactivation in this case is due to conformation changes.

It seems that manipulation of the thiol groups may result in only localized steric or charge effects that perturb the active site of S-GAT. In M-GAT carboxamidomethylation of the thiol groups does not affect the functional properties of the enzyme but MB treatment leads to an overall loss of structure. This concept is strengthened by the following. (a) The shift in the pK of the chromophore by 0.8 unit in S-GAT bound with MB. This is an expected result if the modified sulfhydryl groups are near the active site, or their modification indirectly affects other amino acids near the site (Martinez-Carrion *et al.*, 1970a). In contrast, the pK of the chromophore of M-GAT is completely unaffected by sulfhydryl group modification. (b) The great change in substrate dissociation constant after MB-bound or carboxyamidomethylation of all the sulfhydryl groups in S-GAT. Since the gross structure is retained after these treatments, some steric strain or a change in topography may occur at the active site. The more gradual and smaller changes in the binding of substrates for M-GAT could be due to a lower affinity because of the loss of overall conformational integrity (Table I). (c) The release of pyridoxamine phosphate from S-GAT bound with MB. In the pyridoxamine form of the enzyme, the covalent linkage of the pyridoxal form of the cofactor is now absent so the only stabilizing effects are charge interactions. If after binding of the large MB, unfavorable charge distribution or localized steric strain was exerted at the active site, the pyridoxamine phosphate would tend to dissociate. This is what occurs in S-GAT even though there is no detectable large conformational change. The rate of dissociation from the mitochondrial enzyme is faster and is probably due to the overall conformational changes occurring in this protein after treatment with MB (Table I and Figure 4).

Although sulfhydryl groups may appear to be near the active site at least in S-GAT, they do not significantly affect the chiral environment of pyridoxal phosphate in either isozyme as is apparent from the lack of disturbance of the 430-mμ and 290-mμ ellipticities.

In conclusion, the function of the sulfhydryl groups in the two isozymes is as distinct as are their structures and cytological distribution. In addition to gross conformational changes, topological perturbations can take place. These effects can be potentiated by the bulkier thiol reagents and are more dramatic in the less rigid M-GAT. With respect to sulfhydryl group participation in enzymatic transamination, the problem is now clearer. In M-GAT, it is evident that no thiol group can be implicated in either substrate binding or in catalysis. In S-GAT, thiol groups may not play a catalytic role since the partial losses in catalysis could be explained by localized conformational effects that influence substrate binding.

Acknowledgments

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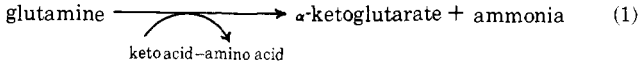
Rat Liver ω-Amidase. Purification and Properties*

Louis B. Hersh

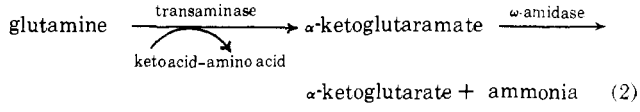
ABSTRACT: The ω-amidase from rat liver has been purified to homogeneity. The native enzyme, mol wt 58,000, can be dissociated into subunits of mol wt 27,000–28,000 by treatment with 7 M guanidine hydrochloride or 7 M urea. A study of the substrate specificity of the enzyme revealed that in addition to hydrolyzing α-ketoglutaramate, glutaramate, and succinamate, the enzyme also hydrolyzes the monomethyl and ethyl esters of α-ketoglutarate (δ esters), succinate, and glutarate. In addition to the hydrolytic reactions the enzyme catalyzes hydroxaminolysis and transamidation with esters and amides. Studies on the nature of the reaction of α-ketoglutaramate as a function of pH revealed that below pH 8, the rate-limiting step of the ω-amidase reaction could be the nonenzymatic conversion of 5-hydroxypyroglutamate into α-ketoglutaramate. This latter finding is discussed in terms of its possible physiological significance.

One of the pathways for the metabolism of glutamine in liver involves the transamination–deamidation of this compound to yield α-ketoglutarate and ammonia (reaction 1).



(1)

Although this system was initially described as a keto acid activated glutaminase (Greenstein and Price, 1949; Errera, 1949), subsequent studies by Meister and coworkers (Meister and Tice, 1950; Meister *et al.*, 1953, 1955; Meister, 1953, 1954) demonstrated that the reaction involves the transamination–deamidation of glutamine rather than a single hydrolytic reaction. Based on the observation that liver preparations would transaminate γ-methylglutamine, but would not deamidate *N*-methyl-α-ketoglutaramate, Meister (1954) proposed the involvement of two enzymes, a transaminase and an ω-amidase, in the overall conversion of glutamine into α-ketoglutarate and ammonia (reaction 2).



(2)

This proposal was supported by the observation that extracts of a mutant of *Neurospora crassa* catalyze the transamination of glutamine, but not the subsequent deamidation of α-ketoglutaramate (Monder and Meister, 1958).

Relatively little work has been done on the characterization of the two enzymes involved in the transamination–deamidation of glutamine. The glutamine transaminase has been purified by Yoshida (1967), and more recently by A. Cooper and A. Meister (in preparation).

The ω-amidase from rat liver has previously been purified 40-fold by Meister (1955). The enzyme was shown to deamidate α-ketosuccinamate, glutaramate, succinaminate, and α-ketoglutaramate. The present paper reports the preparation of rat liver ω-amidase in homogeneous form and a study of its physical and chemical properties.

Materials and Methods

Substrates. α-Ketoglutaramate, α-ketosuccinamate, δ-methyl α-ketoglutarate, and δ-ethyl α-ketoglutarate were pre-

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