Itemizing Enzyme Ligand Interactions in Native and Half-Active Hybrid Aspartate Transaminase to Probe Site–Site Relationships†

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ABSTRACT: A detailed investigation comparing the interaction of ligands with glutamate aspartate transaminase (GAT) modified to the extent that one of its active sites is inactivated by reduction with $NaBH_4$ of the internal aldimine linkage (hybrid) and the native enzyme has been carried out. The experiments provide a direct spectroscopic measure of binary complex formation with a variety of substrates, analogues, and inhibitors with the active-site chromophore in both the pyridoxal and pyridoxamine forms of the enzyme. In this manner, it is possible to detect, using more discriminating techniques than steady-state kinetics, any unusual behavior that may result if cooperative interactions occur during the binding of any of the substrates or inhibitors. The experiments included measurement of the equilibrium binding affinities for the productive complexes of the substrates L-glutamate, L-aspartate, α-ketoglutarate, and oxalacetate as well as the affinities of the substrate analogues *erythro*-β-hydroxyaspartate and α-methylaspartate; in addition, the binding affinities of inhibitory complexes with α-ketoglutarate and chloride were also determined. No significant differences were observed for the formation of binary complexes with the two active sites of dimeric native enzyme and the one active site in dimeric hybrid enzyme. The reaction rate of β,β-difluorooxalacetate with the pyridoxamine form of the enzyme was also studied. The enzyme has a great affinity for this substrate ($K_d \approx 3 \times 10^{-7}$ M) and these studies reveal that a pyridoxamine–anion complex is formed which inhibits the enzyme with K_d (chloride) ≈ 10 mM. The fluorinated substrate analogue was also used to study the selectivity of substrate binding between the pyridoxamine form of the native enzyme and the reduced enzyme when bromophenol blue is utilized as a spectrophotometric probe for anion binding. The results demonstrate that β,β-difluorooxalacetate will bind preferentially to the pyridoxamine form of the enzyme rather than to the reduced active site and that the rates of half transamination, pyridoxal–pyridoxamine form, are identical for both the native or hybrid dimers of the enzyme. Stopped-flow kinetic measurements were also made with the substrate analogue α-methylaspartate. No difference in the rate of formation of the intermediate binary complex absorbing at 430 nm was observed between native and hybrid enzyme. The half-active hybrid also retains the same binding affinity for α-methylaspartate. The experiments were performed over a broad range of protein and substrate concentrations and all the best known binary complexes of the transaminase were looked into (aldimine, ketimine, and semiquinoid, as well as the anion and dicarboxylic acid inhibitor complexes). No difference in the equilibrium constants or rates of reaction between the native and hybrid enzyme exist, as observed by any of the sensitive methods used, and the results are taken as evidence of the independent functioning of the active sites of GAT.

Cooperative behavior between protein subunits in multimeric enzymes has received much attention and numerous examples of such cooperativity have been reported (Stallcup and Koshland, 1973; Koshland, 1970; Janin, 1973). For some of the systems studied, conflicting reports have arisen concerning the existence and type of cooperative behavior (Boettcher and Martinez-Carrion, 1975a; Gutfreund, 1975).

The utilization of chemically modified or genetically altered hybrids is being widely used to attack a variety of problems regarding subunit communication and the strength of subunit interactions. Different chemical hybrid methods have been applied to a number of enzymes, including aldolase (Meighen and Schachman, 1970a), glyceraldehyde-3-phosphate dehydrogenase (Meighen and Schachman, 1970b), glycogen phosphorylase (Feldmann et al., 1976), aspartate transcarbamylase (Gibbons et al., 1975), and tryptophan synthetase (Hathaway et al., 1969). Through the use of genetically altered hybrids, the four active sites of β-galactosidase have been shown to function independently (Melchers and Messer, 1973).

Earlier, we described a novel method for preparing a hybrid enzyme of aspartate transaminase in which, with minor perturbation, one of the active sites of the dimer was inactivated by reduction of the internal aldimine linkage (the ϵ-amino group of lysine-258 and the 4′ carbon of PLP[1]) with $NaBH_4$ (Boettcher and Martinez-Carrion, 1975a). Our initial characterization of the hybrid enzyme demonstrated that no appreciable structural alterations had occurred during the manipulations and the steady-state kinetic parameters obtained at dilute enzyme concentrations indicated that the active sites of GAT may function independently.

Analytical kinetic approaches, which rely on overall substrate consumption or product formation after many enzyme catalytic turnovers, are complex to interpret and can hide subtle deviations from independent catalytic sites behavior. Furthermore, steady-state approaches are unable to detect conditions under which substrates, products, or reaction in-

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[1] Abbreviations used are: GAT, glutamate aspartate transaminase; PLP, pyridoxal 5′-phosphate; Nbs_2, 5,5′-dithiobis(2-nitrobenzoic acid); ANS, 8-anilino-1-naphthalenesulfonate; Tris, 2-amino-2-hydroxymethyl-1,3-propanediol.

TABLE I: Comparison of Binding Properties and Molar Extinction Coefficients of Supernatant Glutamate Aspartate Transaminase after Hybridization.[a]

Compound	Native			Hybrid		
	K_d (mM)	K_m[b] (mM)	ϵ M^{-1} cm^{-1}	K_d (mM)	K_m[b] (mM)	ϵ M^{-1} cm^{-1}
Substrates						
L-Glutamate (K_1)	2.2			2.3		
α-Ketoglutarate (K_3)	0.06	0.09		0.07	0.09	
L-Aspartate (K_1)	0.5	1.9	4 800[c]	0.5	2.0	2 600[c]
Oxalacetate (K_2)	0.001			0.001		
Substrate analogues						
erythro-β-Hydroxyaspartate (K_1)	0.03		50 000[d]	0.03		23 000[d]
α-Methylaspartate (K_1)	0.4		7 400[c]	0.4		3 800[c]
Difluorooxalacetate[e] (K_2)	0.0003			0.0003		
Abortive complex						
α-Ketoglutarate (K_3)	1.0		5 800[c]	0.9		3 100[c]
Anion binding						
Chloride to E_M[f]	~10			~10		

[a] 0.02 M Tris, 0.01 M cacodylate, pH 8.3. [b] See Boettcher and Martinez-Carrion, 1975a. [c] At 430 nm. [d] At 492 nm. [e] 0.01 M pyrophosphate, pH 7.9. [f] Measured as competitive inhibitor of reaction with β,β-difluorooxalacetate.

termediates may bind to an inactive site and, in this manner, exert a cooperative effect on the remaining functional site.

Fortunately, in transaminases there are available a wealth of avenues through which to probe the individual steps of the catalytic reaction and carry out kinetics measurements under single enzyme turnover conditions (Jenkins and D'Ari, 1966a,b,c; Jenkins and Taylor, 1965; Cheng and Martinez-Carrion, 1972). Methods can also be devised for the study of noncatalytically productive complexes between substrates or inhibitors and the active-site chromophore, or between a modified active site and active site directed ligands (Cheng, 1973; Martinez-Carrion et al., 1973, 1976). Thus, we decided that more precise measurements should be used to detect possible hidden interactions between the two sites. The additional studies presented herein give further justification for the independent functioning of the active sites of GAT. These studies have been performed at high enzyme concentrations, close to those at physiological intracellular levels, and over a wide variety of substrate concentrations under both equilibrium and nonequilibrium conditions. In addition, the reaction mechanism of GAT has been studied in its individual parts and this work offers a more critical evaluation of possible cooperative behavior.

Materials and Methods

The preparation of the hybrid enzyme with one of the active sites inactivated by reduction with $NaBH_4$ of the internal aldimine linkage from the α subform of pig heart supernatant glutamate aspartate transaminase has previously been described (Boettcher and Martinez-Carrion, 1975a). The specific activity of the native enzyme was 300–320 μmol min^{-1} mg^{-1} using the coupled malate dehydrogenase assay at pH 7.5, while that of the hybrid was 140–160 μmol min^{-1} mg^{-1}. For the spectrophotometric studies, the enzyme was maintained in a 0.02 M Tris–0.01 M cacodylate buffer, pH 8.3. Deionized enzyme was prepared by passage through a mixed-bed resin after initial dialysis against deionized water (Martinez-Carrion et al., 1973). The pyridoxamine form of the enzyme was prepared with L-cysteinesulfinic acid according to the method of Jenkins (Jenkins and D'Ari, 1966a).

The spectrophotometric studies were performed with a Cary 15 spectrophotometer equipped with a 0–0.1 absorbance scale slide wire using 1-ml quartz cells of 1-cm light path. The reaction of β,β-difluorooxalacetate with the pyridoxamine form of the enzyme was carried out in 10-cm light path cylindrical cells of 30-ml volume. A 10 mM pyrophosphate buffer, pH 7.9, was used and the cell compartment was thermostated at 30 °C. Appearance of the pyridoxal form of the enzyme was monitored at 360 nm and k_{obsd} was calculated by plotting log ΔA_{360} against t. Stopped-flow experiments were done with a Gibson-Durrum stopped-flow spectrophotometer equipped with photometric log amplifier and 2-cm light path with the temperature controlled at 25 °C. The raw data was recorded from the oscilloscope tracing on self-developing film and treated with the equation:

$$\ln C_\beta^\infty - \ln (C_\beta^\infty - C_\beta) = k_{obsd} t$$

where C_β^∞ is the final product concentration and C_β is the product concentration of various time intervals. The reaction with α-methylaspartate was followed at 430 nm at pH 8.2 using the deionized enzyme adjusted with solid Tris to the final pH. The instrument dead time was approximately 4 ms.

The substrate analogue β,β-difluorooxalacetate (Kun et al., 1963) was a gift of Dr. J. C. Slebe. Bromophenol blue was a K & K product.

The extinction coefficients given in Table I are based on the protein concentration of the dimer, rather than the monomer, and, consequently, give values twice those based on the monomer.

In general, the experimental points represent the average of two or more measurements and the estimated error is less than 10% for the experimentally determined binding constants.

Theory and Results

The methods for spectrophotometric titration of the enzyme's active-site chromophore given herein have been published (Jenkins and D'Ari, 1966b,c; Jenkins and Taylor, 1965) and these works should be consulted for further details. Only a summary will be described here for convenience and easy reference.

The spectral changes which occur upon addition of keto (O) and amino (Am) acids to the pyridoxal (E_L) and pyridoxamine (E_M) forms of the enzyme can be described with Scheme I

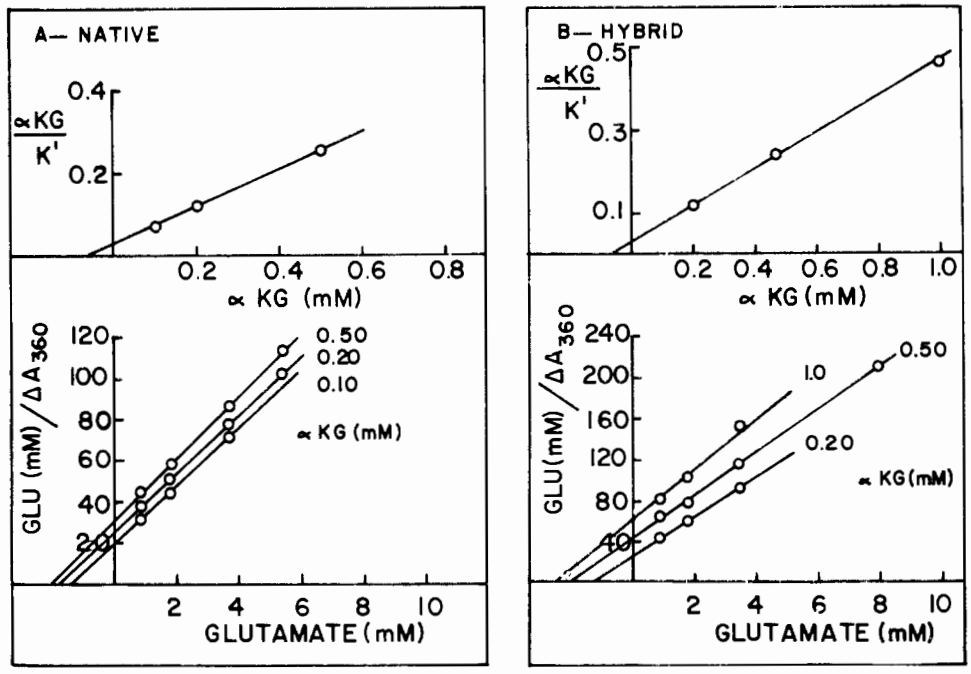

FIGURE 1: Determination of K_1 and K_2 for the substrate pair L-glutamate and α-ketoglutarate. The primary plot of [glutamate]/ΔA_{360} vs. [glutamate] intersects the abscissa at the point $-K' = K_1/(1 + (K_2/[O]))$, which can be expressed by the equation $[O]/K' = ([O]/K_1) + (K_2/K_1)$. The secondary plot of $[O]/K'$ against [O] has an abscissa intercept of $-K_2$ and an ordinate intercept of K_2/K_1.

Scheme I

$$\begin{array}{ccc} E_LO & & E_MAm \\ K_3 \downarrow\uparrow O & & K_4 \downarrow\uparrow Am \\ E_L + Am & \underset{K_1}{\rightleftharpoons} & EX \underset{K_2}{\rightleftharpoons} E_M + O \end{array}$$

(Jenkins and D'Ari, 1966b). In this scheme EX refers to all the intermediate binary complexes, E_LO and E_MAm are nonproductive abortive complexes, and K_1 through K_4 represent the dissociation constants. The total absorbance at a given wavelength is given by the sum:

$$A_\lambda = \epsilon_1[E_L] + \epsilon_2[E_M] + \epsilon_3[E_LO] + \epsilon_4[E_MAm] + \epsilon_5[EX]$$

$$= [EX]\left\{\epsilon_5 + \frac{K_1}{[Am]}\left(\epsilon_1 + \epsilon_3\frac{[O]}{K_3}\right) + \frac{K_2}{[O]}\left(\epsilon_2 + \epsilon_4\frac{[Am]}{K_4}\right)\right\}$$

where ϵ_1 through ϵ_5 are the molar extinction coefficients for the various enzyme species.

Determination of Substrate and Substrate Analogues Dissociation Constants and Extinction Coefficients. For the determination of K_1 and K_2 of the substrate pair L-glutamate and α-ketoglutarate, the following expression (eq 1) has been derived (Jenkins and D'Ari, 1966b), in which it is assumed under the experimental conditions used (high pH and low keto acid concentration) that no abortive complexes are formed, i.e., $[Am]/K_4$ and $[O]/K_3 = 0$.

$$\frac{[Am]}{\Delta A_\lambda} = \frac{1}{E_t}\left[\frac{K_1 + \left(1 + \frac{K_2}{[O]}\right)[Am]}{(\epsilon_1 - \epsilon_5) + (\epsilon_1 - \epsilon_2)\frac{K_2}{[O]}}\right] \quad (1)$$

Under these conditions, this equation is generally applicable for any wavelength monitored. Following the decrease in absorbance at 360 nm proves to be the most sensitive wavelength. It is necessary to use such an analysis, since there is no unique absorption maximum which can be assigned to the intermediary binary complex. By varying the glutamate concentration at several constant levels of α-ketoglutarate and plotting [glutamate]/ΔA_{360} vs. [glutamate], a series of straight lines is obtained from which it is possible to determine K_1 and K_2. Measurement of K_1 and K_2 for the 5-carbon pair L-glutamate and α-ketoglutarate is shown in Figure 1 and Table I. For the native enzyme, the primary plot of [glutamate]/ΔA_{360} against [glutamate] yields a series of parallel lines, as expected, and the secondary plot indicates a good reliability. The slight deviation from parallel primary plots for the hybrid is the result of using an α-ketoglutarate concentration which was too close to the value of K_3 of α-ketoglutarate. Nonetheless, the secondary plots again show a good reliability.

To determine K_1 and K_2 for the substrate pair L-aspartate and oxalacetate, only the amino acid need be added to the pyridoxal form of the enzyme (Jenkins and Taylor, 1965). This is possible because addition of L-aspartate to the pyridoxal form of the enzyme results in the formation of an equilibrium mixture of the various binary complexes, one of which absorbs at 430 nm. Also, since oxalacetate has a very high affinity for the pyridoxamine enzyme, it is necessary to add the amino acid only in order to obtain significant amounts of the 430-nm absorbing complex, which is a convenient wavelength to follow the formation of the intermediary binary complex since no other interfering absorbing intermediates are detected at this wavelength. If the experimental conditions are chosen such that ϵ_1 and $\epsilon_2 = 0$, i.e., both the pyridoxal and pyridoxamine enzyme forms do not absorb at the measuring wavelength (pH 8.3, 430 nm), the following equation (eq 2) may be used to analyze the data (Jenkins and Taylor, 1965):

$$\frac{E_t}{\Delta A_\lambda} = \frac{1 + \frac{K_1}{[Am]}}{\epsilon_5} + \frac{K_2\left(1 + \frac{[Am]}{K_4}\right)}{\epsilon_5 \times \Delta A_\lambda} \quad (2)$$

and a plot of $E_t/\Delta A_\lambda$ vs. $\sqrt{1/\Delta A_\lambda}$ will yield a series of straight

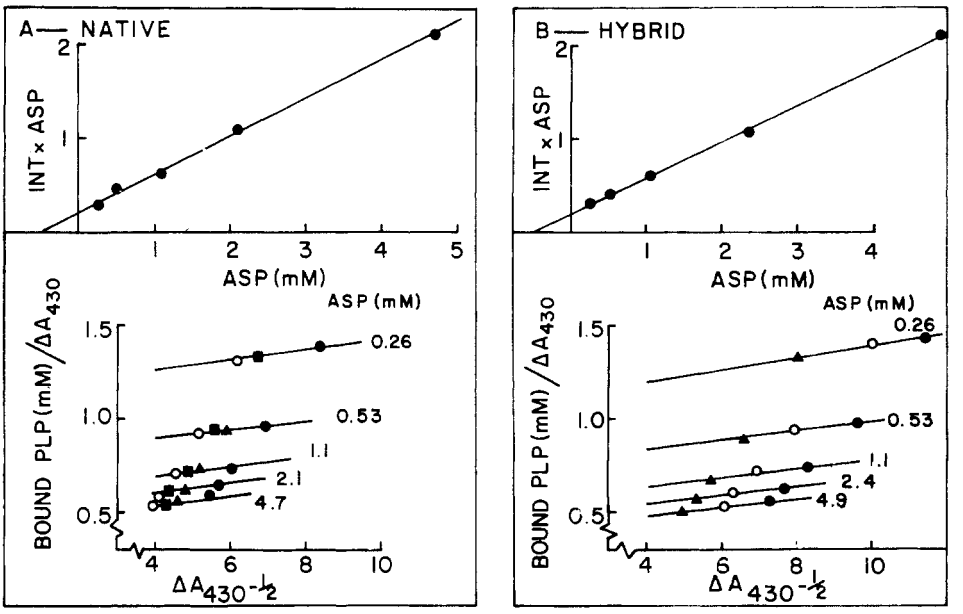

FIGURE 2: Determination of K_1 and K_2 for the substrate pair L-aspartate and oxalacetate. (A) Native: [bound PLP] = (●) 2×10^{-5} M, (△) 2.5×10^{-5} M, (□) 3×10^{-5} M, (○) 3.5×10^{-5} M; (B) hybrid: [bound PLP] = (●) 1×10^{-5} M, (○) 1.5×10^{-5} M, (△) 2.1×10^{-5} M.

lines whose ordinate intercept can be replotted to obtain both K_1 and ϵ_5. If the series of lines from the primary plot are parallel, this implies that K_4 is much larger than the amino acid concentrations used and thus E_MAm is negligible. This being the case, one can obtain K_2 from the slope of any of the lines knowing ϵ_5.

Figure 2 shows these plots for aspartate with the native and hybrid enzyme forms. The primary plot of bound PLP (mM)/ΔA_{430} vs. $\sqrt{1/\Delta A_{430}}$ obtained by titrating varying concentrations of the enzyme with different aspartate levels yields a series of straight lines of constant slope. The secondary plots of the ordinate intercept × [L-aspartate] vs. [L-aspartate] are also linear in both cases and give $1/\epsilon_5$ as slope and $-K_1$ as the negative abscissa intercept. The value of K_2 is obtained from the constant slope of the primary plot $E_t/\Delta A_{430}$ vs. $\sqrt{1/\Delta A_{430}}$ and any error in this slope is magnified by being squared, thus giving less reliable values. Overall, K_1 and K_2 agree well, as do the extinction coefficients for the intermediate complex absorbing at 430 nm (Table I).

In a similar manner, one can also obtain K_1 and ϵ_5 for the substrate analogue *erythro*-β-hydroxyaspartate (Table I). Addition of hydroxyaspartate to the pyridoxal enzyme results in the formation of the pyridoxamine enzyme and the corresponding keto acid, as well as an intermediary binary complex, with a unique absorption maximum at 490 nm. This absorption maximum corresponds to the semiquinoid binary intermediate in the transaminase scheme.

For the determination of K_1 for the substrate analogue α-methylaspartate, which forms only the initial binary complexes with the pyridoxal form of the enzyme, the following reaction is considered

$$E + S \overset{K_1}{\rightleftharpoons} ES$$

since the substrate analogue lacks the α proton and transamination is not possible. The data can be analyzed with the following equation (eq 3) (Michuda and Martinez-Carrion, 1969b).

$$\frac{[E_t]}{\Delta A_{430}} = \left(\frac{K_1}{(\epsilon_2 - \epsilon_1)}\right)\frac{1}{[S]} + \frac{1}{(\epsilon_2 - \epsilon_1)} \qquad (3)$$

In this case, ϵ_1 is the extinction coefficient of the free pyridoxal form of the enzyme and ϵ_2 represents that for the enzyme–substrate complex. Plotting $1/\Delta A_{430}$ vs. $1/[\alpha$-methylaspartate] gives a straight line from which K_1 and ϵ_2 are obtained (ϵ_1 being nearly zero at high pH and 430 nm). The K_1 and ϵ_2 values for both the native and hybrid enzymes are listed in Table I.

For the determination of K_3, the dissociation constant of the abortive complex of α-ketoglutarate with the pyridoxal form of the enzyme, the same analysis is applied as for binding of α-methylaspartate to the pyridoxal enzyme (Michuda and Martinez-Carrion, 1969a). Table I lists the experimental values for K_3 and ϵ_2 of the native and hybrid enzymes.

Stopped-Flow Analysis of α-Methylaspartate Binding. The addition of the substrate analogue α-methylaspartate to the pyridoxal enzyme results in the formation of a binary intermediary complex absorbing at 430 nm, while, at the same time, showing a decrease in absorbance at 360 nm as the pyridoxal enzyme reacts with the amino acid. The kinetics of formation of this binary complex is another important experimental measure of the transaminase reaction, quite different from either steady-state kinetics or equilibrium binding studies.

The rapid mixing and subsequent reactions of the pyridoxal form of the enzyme with α-methylaspartate followed at either 360 or 430 nm may conveniently be described with the following reaction (Cheng, 1973).

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES_1 \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} ES_2$$

Here E is the pyridoxal form of the enzyme, while S is the substrate analogue α-methylaspartate which is in large excess over the enzyme concentration. If k_2 is large with respect to k_{-2}, the following expression may be applied to analyze the system (Cheng, 1973):

$$k_{obsd} = \frac{k_2 S}{K_d + S} \qquad (4)$$

where

$$K_d = \frac{k_2 + k_{-1}}{k_1} \qquad (5)$$

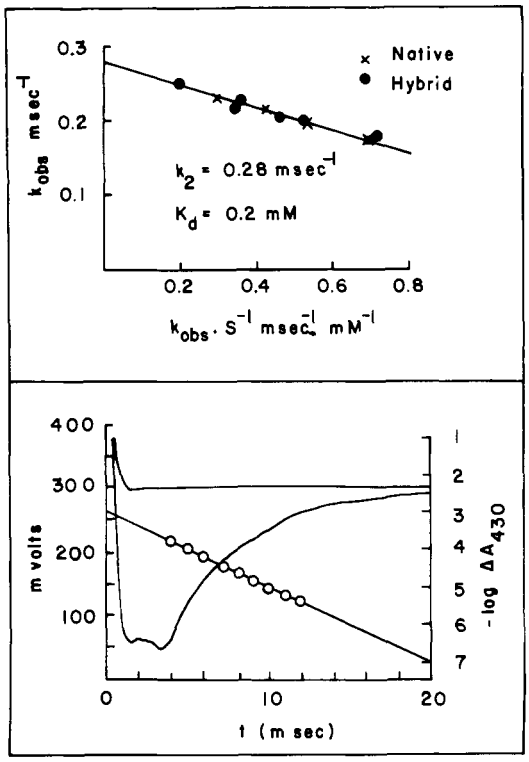

FIGURE 3: Stopped-flow analysis of α-methylaspartate binding.

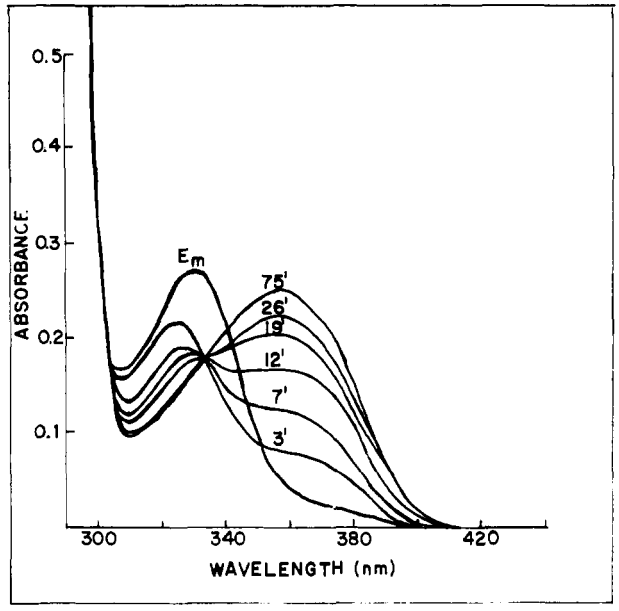

FIGURE 4: Time dependence of the effect of addition of β,β-difluorooxalacetate on the absorption spectrum of the pyridoxamine form of the enzyme.

and a plot k_{obsd} vs. k_{obsd}/S has a slope of $-K_d$ and an ordinate intercept of k_2. Figure 3 shows this plot for both the native and hybrid species. The data essentially was the same for both enzyme species with K_d = 0.2 mM and k_2 = 0.28 ms^{-1}. The figure also shows the raw data and its treatment to obtain k_{obsd}. The K_d value of 0.2 mM from the stopped-flow data was lower than expected but still demonstrates the identical behavior of the native and hybrid species.

Reaction of β,β-Difluorooxalacetate with the Pyridoxamine Form of the Enzyme. The reaction of the pyridoxamine enzyme with a keto acid is an important aspect of the transaminase reaction. However, this reaction is generally quite fast with the usual keto acids and thus difficult to follow. The reaction with pyruvate is relatively slow and can be followed in the stopped-flow spectrometer (Cheng, 1973). However, the dicarboxylic acid β,β-difluorooxalacetate (Kun et al., 1963) may be a better substrate analogue to follow this reaction (Briley et al., 1973; Jenkins, 1963).

When β,β-difluorooxalacetate is added to the pyridoxamine enzyme, one observes the rapid initial formation of the ketimine binary complex (~5-nm shift to shorter wavelength from pyridoxamine absorption) and subsequent relatively slow formation of the aldimine form of the enzyme (360-nm absorption) (Figure 4).

The time course of this reaction can conveniently be monitored at 360 nm. Scheme II illustrates some possible interac-

Scheme II

$$E_M \underset{(O)}{\overset{K_d}{\rightleftharpoons}} EX \xrightarrow{k_2} E_L + Am$$

$$K_{An} \Big\| (An)$$

$$E_MAn$$

tions which may occur in this system, where

$$k_{obsd} = \frac{k_2 E_t(O)}{K_d' + (O)} \qquad (6)$$

and

$$K_d' = K_d\left(1 + \frac{An}{K_{an}}\right) \qquad (7)$$

Included in this scheme is the competitive interaction of an anion (An) and β,β-difluorooxalacetate (O) with the pyridoxamine (E_M) enzyme. The appearance of the pyridoxal enzyme (E_L) will follow first-order kinetics with respect to (EX), which, in turn, is dependent upon the concentration of An, E_M, and O. By holding [E_M] constant (1 × 10^{-6} M) and varying the concentrations of chloride (0.1–0.4 M) and β,β-difluorooxalacetate (4.3 × 10^{-6} M to 3.4 × 10^{-5} M), the double-reciprocal plots (Figure 5) of the inverse observed rate against the inverse of the substrate concentration are competitive for chloride for both the native and hybrid species and give close values for the maximum rate (ordinate intercept).

The calculated second-order rate constants are 1.0 × 10^5 M^{-1} min^{-1} for the native species and 1.2 × 10^5 M^{-1} min^{-1} for the hybrid enzyme. The measured bimolecular rate constant is higher than the value previously reported by Briley et al. (1973) but this could be the result of the anion effect which was overlooked by these authors. The bimolecular rate constants for the native and hybrid enzymes are close and both agree with the value obtained at saturating substrate levels for the deionized enzyme. Chloride anion behaved in a competitive fashion with the substrate analogue. The secondary plot of the apparent dissociation constant (obtained from the negative abscissa intercept as $1/K_d$) vs. chloride concentration gives a value of 3 × 10^{-7} M as the true K_d of difluorooxalacetate (ordinate intercept) and approximately 10 mM as the K_d of chloride for the pyridoxamine enzyme (negative abscissa intercept). Although the value for the dissociation constant of chloride for the pyridoxamine enzyme is only an approximate value, it agrees well with other measured values (Martinez-Carrion et al. (1973).

Displacement of Bromophenol Blue Anion with β,β-Difluorooxalacetate from the Pyridoxamine Enzyme. The binding of anions may be followed by their competition for substrate binding. In addition, if the anion exhibits a property, such as a distinct absorption spectrum in the free and bound

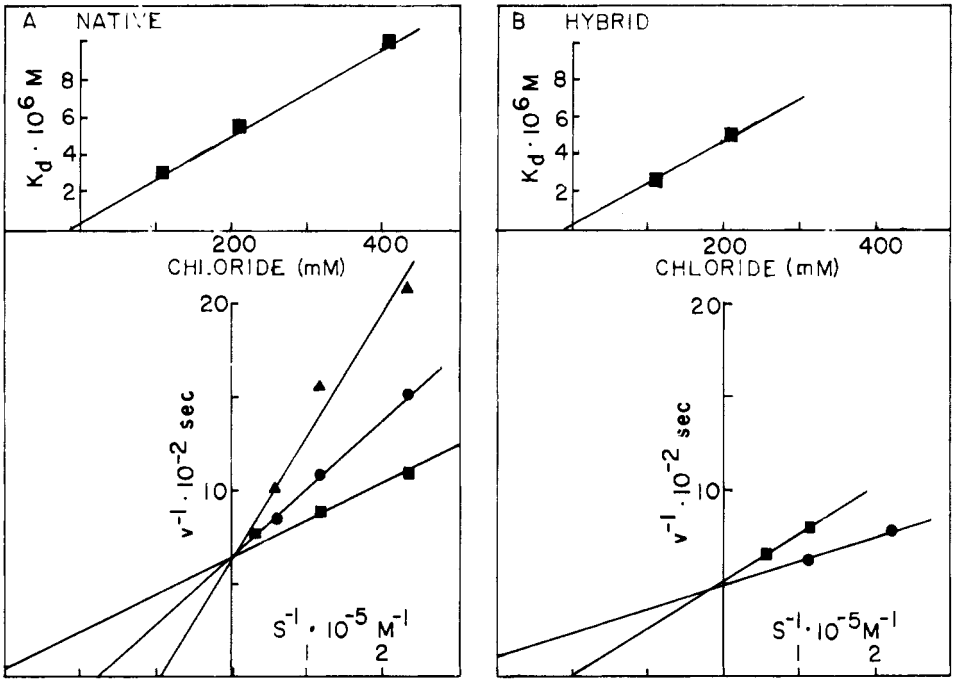

FIGURE 5: Graphic kinetic analysis of the reaction of β,β-difluorooxalacetate with the pyridoxamine enzyme. (A) Native: (■) 100 mM chloride, (●) 200 mM chloride, (▲) 400 mM chloride; (B) hybrid: (●) 100 mM chloride, (■) 200 mM chloride.

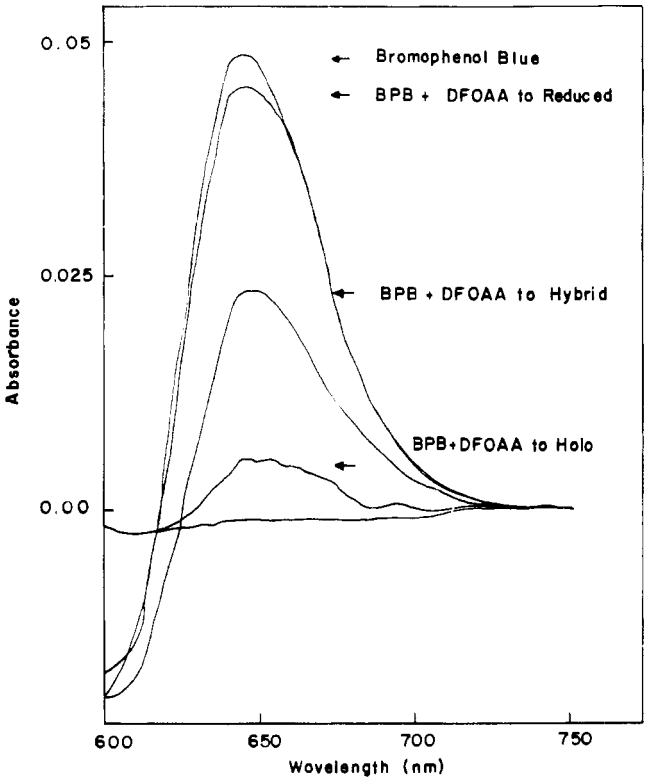

FIGURE 6: Difference spectra of bromophenol blue binding to the native, hybrid, and reduced deionized enzymes showing effect of addition of saturating amounts of β,β-difluorooxalacetate (DFOAA). Conditions: 3×10^{-7} M deionized enzyme, pH 8; $0.9\text{–}3.8 \times 10^{-6}$ M bromophenol blue; 4.5×10^{-6} M β,β-difluorooxalacetate ($\sim 15 \times K_d$). Holo refers to native holoenzyme.

state, it is possible to directly monitor the binding of the anion on the protein. Monitoring of anion binding in this manner is of importance, since this provides direct insight into the anion-binding process.

The binding of the dye bromophenol blue to the anion binding site results in a bathochromatic shift of the maximum absorption of the dye to 650 nm (Cheng, 1973; Harruff and Jenkins, 1976). By recording the visible difference spectrum, the relative amounts of free and bound bromophenol blue can be determined. If bromophenol blue is enzyme bound and the substrate is added, a decrease in the 650-nm absorption is observed as bromophenol blue is displaced by the substrate. Figure 6 shows these difference spectra for various deionized forms with and without saturating amounts of β,β-difluorooxalacetate. Very little displacement is shown for the reduced form, while the native enzyme (pyridoxamine form) exhibits nearly full displacement and the hybrid (half-reduced, half pyridoxamine) shows an intermediate value. Attempts to follow this displacement kinetically in the stopped-flow spectrometer with addition of α-methylaspartate to the deionized enzyme proved unsuccessful, as the displacement was too fast. This may indicate that the displacement occurs on the same time scale ($t_{1/2} \approx 0.1$ ms) as proton uptake by the active site histidine (Giannini et al., 1975). This is the histidine which binds anions and dicarboxylic acids (Peterson and Martinez-Carrion, 1970; Cheng and Martinez-Carrion, 1972; Martinez-Carrion et al., 1973).

Discussion

Prior to our preparation of a hybrid enzyme, a number of experiments had been interpreted as favoring cooperative behavior between the subunits of GAT. The possibility of negative cooperativity in this enzyme was originally introduced by Christen and Riordan (1970). Their assumption was based on the observation that tetranitromethane modified only a single tyrosine per dimer with full loss of activity. However, it was later shown that this was a secondary reaction following inactivation due to modification of sulfhydryl groups (Birchmeier et al., 1973). Data on the coenzyme binding to the apoenzyme has also been contradictory (Arrio-Dupont, 1972; Churchich and Farrelly, 1969; Lee and Churchich, 1975; Schlegel and Christen, 1974), indicating both normal and negative coop-

erative binding. Other experiments involving the use of protein-modifying or fluorescent reagents, such as Nbs_2 and ANS (Cournil and Arrio-Dupont, 1973; Harris and Bayley, 1975), were interpreted as cooperative behavior without much regard given to the possibility that insertion of these probes could either produce asymmetry upon reaction or binding, or, in the case of ANS binding, be localized at the subunit interface. More recently, the nonlinear binding of phthalate has been suggested as evidence that the substrate binding might follow some possible cooperative behavior (Bayley and Harris, 1975). However, it is known that the carbon backbone is important in binding dicarboxylic acids (Bonsib et al., 1975) and exchange of a flexible hydrocarbon chain for a bulky, rigid aromatic group may produce significant alterations in binding behavior. Similarly, the apparent nonlinear reactivity with Nbs_2 of the thiol groups in the apoenzyme in the presence of varying amounts of PLP and the apparent lack of thiol reactivity of an apo/holo hybrid have also been used in support of a mode of cooperativity between subunits (Arrio-Dupont and Cournil, 1975). The large number of experiments generated in the search for subunit communication and the resulting confusion have necessitated our divergent path to define the existence of the apparent cooperativity.

The purpose of the various experiments with the hybrid and native enzyme forms has been to test, in a rigorous fashion, the existence of subunit interplay between two active sites of the transaminase. Table I summarizes the results for most of these experiments, where, under our experimental conditions, the enzyme remains a dimer (Feliss and Martinez-Carrion, 1970). As seen, no significant differences are apparent. The results of these experiments cannot always be compared directly with other published results, since we used buffers with a weakly binding competitive anion (10 mM cacodylate), and the dissociation constants reported are closer to the values for the deionized enzyme. Likewise, the ratio K_1:K_2 for both L-glutamate/α-ketoglutarate and L-aspartate/oxalacetate are higher than earlier reported (Jenkins and D'Ari, 1966b; Jenkins and Taylor, 1965). This is also a result of the anion which affects more strongly binding to the pyridoxamine enzyme than the pyridoxal enzyme (Cheng et al., 1971, and Martinez-Carrion et al., 1973).

The results of the competitive effect of chloride on the reaction of β,β-difluorooxalacetate with the pyridoxamine form of the enzyme and the binding of bromophenol blue to the pryridoxamine enzyme give further proof for the existence of a pyridoxamine enzyme–anion complex (Cheng et al., 1973) and that formation of this complex is not impaired in the hybrid enzyme.

Evidence is also presented regarding the lack of binding of the substrate to the reduced coenzyme site. Bromophenol blue is a high affinity anion ($K_d \approx 3 \times 10^{-6}$ M) for the transaminase, which binds with the same stoichiometry as other anions and apparently to the active-site histidine (Cheng, 1973). As with other anions, it is displaced by the substrate in a mutually exclusive fashion (Cheng, 1973). The data in Figure 6 shows the bromophenol blue displacement from the pyridoxamine enzyme using an excess of β,β-difluorooxalacetate. This demonstrates that this substrate analogue shows preference for the nonreduced enzyme site in displacing only bromophenol blue from the pyridoxamine enzyme. Even at saturating levels of the substrate (i.e., $15 \times K_d$), no displacement of bromophenol blue by the substrate is observed from the reduced coenzyme site. This evidence corroborates earlier NMR work from this laboratory in which a different anion, trifluoroacetate, was readily displaced by α-methylaspartate from the native enzyme but not from the reduced enzyme (Cheng and Martinez-Carrion, 1973). It would thus appear quite likely that when experimenting with substrate concentrations in the region of their K_d value, only the nonreduced site will be occupied by the substrate. This would minimize any effects caused by the presence of the substrate on the reduced site, which could, in turn, make it difficult to distinguish interacting and noninteracting sites in hybrid systems.

Most cooperative behavior in enzymes is usually detected at the level of substrate and/or modifier binding. However, deviation of independence of the sites after formation of the enzyme substrate complexes can also occur. In the transaminases, this possibility is tested by measuring the rate of formation of the enzyme's binary complexes with α-methylaspartate and the rates of transformation of the pyridoxamine to the pyridoxal enzyme by reaction with the keto acid β,β-difluorooxalacetate. No significant differences are detected in either case between the native dimer and the hybrid enzyme. These findings, together with the steady-state kinetic results previously reported (Boettcher and Martinez-Carrion, 1975a), minimize the possibility of a dynamic difference, natural or artificially induced by the mild chemical modification of $NaBH_4$ reduction, between the catalytic steps of the native and hybrid enzymes. The lack of evidence for a one-site dimeric enzyme behaving any different from the two site enzyme reported in this work is in accord with NMR results that have failed to reveal preexisting asymmetry in the native dimer with ^{13}C (Boettcher and Martinez-Carrion, 1975b), ^{31}P (Martinez-Carrion, 1975), and ^{19}F (Martinez-Carrion et al., 1973; Cheng and Martinez-Carrion, 1973; Martinez-Carrion et al., 1976; Critz, 1976) NMR probes, even though ^{19}F has been shown to be an extremely sensitive probe for environmental differences (Huestis and Raftery, 1975; Hull and Sykes, 1974).

The present studies reveal no significant difference between the native and hybrid enzymes at any of the control points studied in the transaminase mechanism. From the present evidence, we must conclude that no active-site interactions occur with the transaminase in ligand binding or one turnover transformations of the enzyme in a half transamination sequence and that the mechanistic or structural reasons for the dimeric existence of GAT still remain to be identified.

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