Hybridization of Glutamate Aspartate Transaminase. Investigation of Subunit Interaction†

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ABSTRACT: Glutamate aspartate transaminase (EC 2.6.1.1) is a dimeric enzyme with identical subunits with each active site containing pyridoxal 5′-phosphate linked via an internal Shiff's base to a lysine residue. It is not known if these sites interact during catalysis but negative cooperativity has been reported for the binding of the coenzyme (Arrio-Dupont, M. (1972), *Eur. J. Biochem. 30,* 307). Also nonequivalence of its subunits in binding 8-anilinonaphthalene-1-sulfonate (Harris, H. E., and Bayley, P. M. (1975), *Biochem. J. 145,* 125), in modification of only a single tyrosine with full loss of activity (Christen, P., and Riordan, J. F. (1970), *Biochemistry 9,* 3025), and following modification with 5,5′-dithiobis(2-nitrobenzoic acid) (Cournil, I., and Arrio-Dupont, M. (1973), *Biochemie 55,* 103) has been reported. However, steady-state and transient kinetic methods as well as direct titration of the active site chromophore with substrates and substrate analogs have not revealed any cooperative phenomena (Braunstein, A. E. (1973), *Enzymes, 3rd Ed. 9,* 379). It was therefore decided that a more direct approach should be used to clarify the question of subunit interaction during the covalent phase of catalysis. To this end a hybrid method was devised in which a hybrid transaminase was prepared which contained one subunit with a functional active site while the other subunit has the internal Shiff's base reduced with $NaBH_4$. The specific activities and amount of "actively bound" pyridoxal 5′-phosphate are both in a 2:1 ratio for the native and hybrid forms. Comparison of the steady-state kinetic properties of the hybrid and native enzyme forms shows that both forms give parallel double reciprocal plots which is characteristic of the Ping-Pong Bi-Bi mechanism of transamination. The K_m values for the substrates L-aspartic acid and α-ketoglutaric acid are nearly identical while the V_{max} value for the hybrid is one-half the value of the native transaminase. It therefore appears that the active sites of glutamate aspartate transaminase function independently and a compulsory flip-flop mechanism is not involved.

The general problem of whether there is subunit interplay in multiple subunit enzymes has been observed in various manners such as nonequivalent ligand binding constants (Conway and Koshland, 1968; Lazdunski et al., 1971), differences in reactivity of amino acid residues (Stallcup and Koshland, 1973), and with steady-state and transient kinetics (Harada and Wolfe, 1968; Chappelet-Tordo et al., 1974). In certain cases such as glyceraldehyde-3-phosphate dehydrogenase (Conway and Koshland, 1968; Stallcup and koshland, 1973) there is little doubt as to the existence of this subunit interplay. However, for a variety of other enzymes such as alkaline phosphatase (Bloch and Schlesinger, 1973; Lazdunski et al., 1971; Chappelet-Tordo et al., 1974), malate dehydrogenase (Harada and Wolfe, 1968; Cassman and Vetterlein, 1974; Koren and Hammes, 1975), and alcohol dehydrogenase (Bernhard et al., 1970; Shore and Gutfreund, 1970) there are conflicting reports. For glutamate aspartate transaminase the same controversy has arisen as observed by negative cooperativity in the coenzyme binding (Arrio-Dupont, 1972), nonequivalence of its subunits in binding 8-anilinonaphthalene-1-sulfonate (Harris and Bayley, 1975), in modification of a tyrosine residue (Christen and Riordan, 1970), and following modification with 5,5′-dithiobis(2-nitrobenzoic acid) (Cournil and Arrio-Dupont, 1973). However, other methods have not revealed any cooperative interactions between the subunits of glutamate aspartate transaminase (Braunstein, 1973).

It was, therefore, decided that an independent approach was needed to study possible subunit interactions in enzymes which can be easily labeled at the active site. The approach is that of preparation of active-site modified hybrids.

The preparation of hybrids of native and chemically modified enzymes to study subunit structure and the strength of subunit interactions in multiple subunit proteins has been successfully accomplished by Meighen and Schachman (1970a,b). We have modified this elegant experimental concept by preparing hybrids in which the chemical modification occurs at the active site with loss of catalytic activity in the modified subunit. The procedure of chemical inactivation in dimeric glutamate aspartate transaminase holoenzyme can be achieved with a minimum of protomer conformational alteration by reduction of the internal Shiff's base between coenzyme and enzyme with $NaBH_4$. Subsequent dissociation of the reduced enzyme and recombination with dissociated native enzyme should permit the preparation of dimeric hybrids in which one active site is modified and the other remains active.

Materials and Methods

The α form of supernatant glutamate aspartate transaminase from pig heart was prepared as previously described (Martinez-Carrion et al., 1967; Jenkins and D'Ari, 1966a). To form the S-(α,β-dicarboxyethyl) derivative, the enzyme (5 mg/ml) was reacted in a 0.05 M maleate–0.005 M

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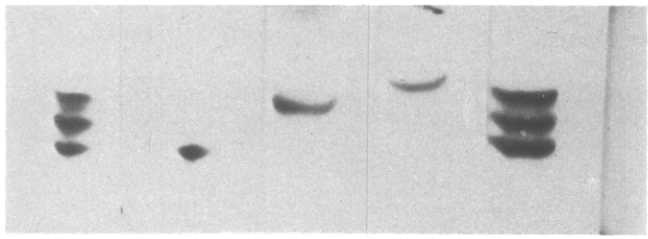

FIGURE 1: Polyacrylamide disc gel electrophoresis showing the separation of the enzyme forms using the pH 9.5 disc gel system. From right to left in the figure the first gel represents the mixture of the three species before separation with CM-Sephadex. The second, third, and fourth gels respectively represent the native, the hybrid, and the $NaBH_4$-reduced S-(α,β-dicarboxyethyl) derivative. The gel farthest to the left is a mixture of the three species after separation on CM-Sephadex and after being remixed.

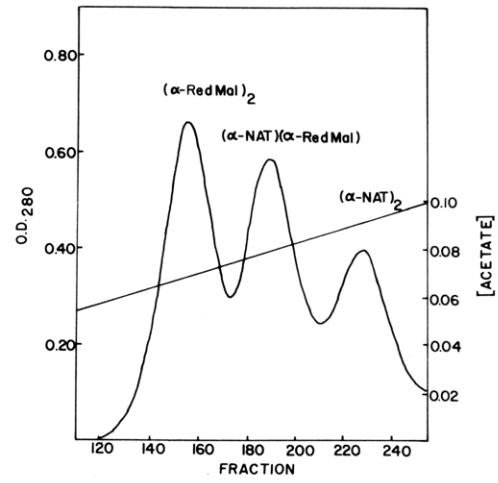

FIGURE 2: Elution profile of the mixture of enzyme species on CM-Sephadex using a linear gradient of 0.02–0.10 M acetate (pH 5.4).

EDTA[1] (pH 6.0) buffer with 0.005 M α-ketoglutarate at 75°C for 30 min (Turano et al., 1964). The S-(α,β-dicarboxyethyl) derivative was then reacted with a 100-fold molar excess of $NaBH_4$ in 0.10 M potassium phosphate (pH 7.5) and dialyzed extensively against water giving the reduced form of the enzyme. Protein concentration was determined at 280 nm using ϵ_{280} 1.3×10^5 M^{-1} cm^{-1} and a molecular weight of 94,000 for the dimer. Steady-state kinetics and enzyme assays were performed on a Cary 15 spectrophotometer at 25°C using the coupled malate dehydrogenase assay. The reaction mixture included 20 units (micromoles of NADH oxidized per minute per milligram of enzyme) per ml of malate dehydrogenase and 0.2 mM NADH. Routine assays were done in 0.05 M sodium phosphate buffer (pH 7.5) with 0.1 M L-aspartate and 6 mM α-ketoglutarate. Measurements of pH were determined with a Radiometer PHM 26 equipped with a GK 2302C combination electrode.

Preparation and Separation of the Hybrid. Polyanovskii et al. (1970) has suggested conditions for dissociating the subunits of glutamate aspartate transaminase. However, it was necessary to modify this method in order to dissociate and reassociate the subunits with only a minor loss of activity. Equal amounts of the α form of the transaminase and the $NaBH_4$ reduced S-(α,β-dicarboxyethyl) derivative were mixed at 4°C in a 12.5 mM glutarate–1.25 mM EDTA, pH 6.0 buffer. A freshly prepared solution of 10 M urea previously passed through a Bio-Rad AG 501-X8 mixed bed resin was added to the enzyme (final concentration 5 mg/ml) giving a final urea concentration of 1 M. The pH of the solution was adjusted to 11.65 for 5 min and then neutralized (to approximately pH 8.5) with 2 M sodium acetate (pH 5.2), 10^{-4} M PLP, and 10^{-2} M α-ketoglutarate before dialyzing against 0.025 M Tris-Cl (pH 8.0) and 10^{-4} M dithiothreitol. The hybrid was then separated on a CM-Sephadex column using a linear gradient of 0.02–0.10 M sodium acetate (pH 5.4).

Results

Reaction of glutamate aspartate transaminase with maleate to form the S-(α,β-dicarboxyethyl) derivative gives a single enzyme species. This derivative has the same specific activity as the native enzyme but has two additional carboxyl groups on each monomer. As shown in Figure 1 this derivative can easily be distinguished from the native form using polyacrylamide disc gel electrophoresis. Using the pH 9.5 disc gel electrophoresis system, the S-(α,β-dicarboxyethyl) derivative has the greatest mobility. Also shown in the figure is a protein band with a mobility intermediate to the native and modified enzyme forms. This band corresponds to the hybrid enzyme where one subunit is the native species while the other subunit is the S-(α,β-dicarboxyethyl) derivative.

Since the subforms of glutamate aspartate transaminase have been separated using a salt gradient on CM-Sephadex (Martinez-Carrion et al., 1967), this type of column appeared to be well suited for isolation of the hybrid enzyme form. Figure 2 shows the separation of the hybrid species on CM-Sephadex at pH 5.4. Again because of the charge differences of the native and S-(α,β-dicarboxyethyl) derivative the hybrid species can be readily separated from the nonmodified and fully modified enzyme forms.

PLP at the active site of glutamate aspartate transaminase has its characteristic spectral properties (Braunstein, 1973). These properties are due to the aldimine (or Shiff's base) linkage between the carbonyl group of PLP and the ϵ amino group of lysine-258 (Braunstein, 1973). The protonated aldimine shows a strong absorbance at 430 nm while the nonprotonated form absorbs at 360 nm. When the Shiff's base is reduced with $NaBH_4$ this pH dependence is lost and a strong absorbance appears only at 335 nm. Figure 3 shows the spectrum for each of the possible enzyme forms at pH 5.2 when the aldimine linkage is protonated. Proceeding from the native, to the hybrid, to the fully reduced enzymes there is a constant decrease in the amount of 430-nm absorbing species with a corresponding increase in the amount of 335-nm absorbing species. Table I summarizes the results of the hybridization in terms of the "actively bound" PLP content (430-nm absorbing species) and specific activity. Both the amount of "actively bound" PLP and specific activities of the native and hybrid are in a 2:1 ratio. In addition, the native enzyme, after being subjected to the dissociating conditions, exhibited only a slight loss of activity when compared to the control.

To determine whether the enzymatic activity measured under conditions of high substrate concentrations represent true V_{max} values and that the substrate affinities have not changed, the steady-state kinetics for both the native and

[1] Abbreviations used are: PLP, pyridoxal 5′-phosphate; EDTA, ethylenediaminetetraacetic acid; Tris, tris(hydroxymethyl)aminomethane; α-Red Mal, $NaBH_4$ reduced, S-(α,β-dicarboxyethyl) derivative of the α subform of glutamate aspartate transaminase.

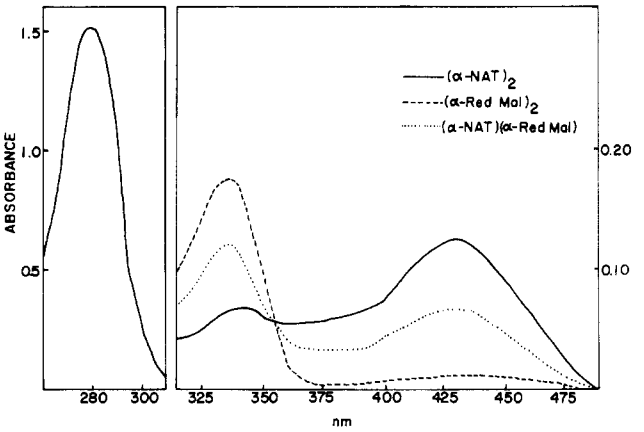

FIGURE 3: Absorption spectra of the three enzyme species at pH 5.2 after CM-Sephadex separation. The spectra are normalized to give the same absorbance at 280 nm.

Table I: Results of Hybridization of Supernatant Glutamate Aspartate Transaminase.

Compound	Absorbance Ratio[a] A_{280}/A_{430}	Absorbance Ratio[a] $A_{280}/A_{335(340)}$	Specific Activity (μmol per min per mg of Enzyme)
α-Control[b]	11	33	330
$(\alpha\text{-NAT})_2$[c]	12	21	320
(α-NAT) (α-Red Mal)	23	13	160
$(\alpha\text{-Red Mal})_2$	140	8.7	20

[a] At pH 5.2. [b] α-Control is either α-glutamate aspartate transaminase or S-(α,β-dicarboxyethyl)-α-glutamate aspartate transaminase. [c] α-NAT, nonmodified α-glutamate aspartate transaminase.

hybrid enzyme forms were measured. Figure 4 shows the steady-state kinetic double reciprocal plots of the native and hybrid forms. In these experiments the concentration of L-aspartate was held constant while the α-ketoglutarate concentration was varied. The ordinate intercept represents an apparent V_{max} while the slope represents the ratio of the K_m of α-ketoglutarate to the V_{max}. Both species give parallel double reciprocal plots characteristic of the Ping-Pong Bi-Bi mechanism for transamination (Henson and Cleland, 1964; Cleland, 1963a,b). The secondary plots, obtained by plotting the ordinate intercepts in Figure 4 against the reciprocal of the L-aspartate concentration, give a true V_{max} of 310 units (μmol/min) per mg for the native fraction and a V_{max} of 160 units/mg for the hybrid. The K_m of L-aspartate for the native form was 1.9 m*M* while the hybrid had a value of 2.0 m*M*. The K_m of α-ketoglutarate for the native form was 0.091 m*M* while the hybrid had a value of 0.094 m*M*.

Discussion

To be able to conveniently separate the hybrid enzyme with ion exchange chromatography and monitor its formation with polyacrylamide disc gel electrophoresis, the α subform of glutamate aspartate transaminase was reacted with maleate to form the S-(α,β-dicarboxyethyl) derivative. This derivative has the same specific activity as the native enzyme. Steady-state kinetic analysis (Henson and Cleland, 1964) comparing the "anionic isozyme" (S-(α,β-dicarboxyethyl) derivative) with the "cationic isozyme" (native enzyme) showed that the substrate and inhibitor parameters were nearly identical for both forms. Also, direct measurements of substrate and substrate analog affinities have revealed no differences in the two forms (Jenkins et al., 1959; Jenkins, 1961a,b, 1964; Jenkins and Taylor, 1965; Jenkins and D'Ari, 1966a; Michuda and Martinez-Carrion, 1969, 1970). The reduction of the internal Shiff's base with $NaBH_4$ is a mild procedure which completely inactivates the enzyme. The reduced enzyme has similar overall structure as determined by circular dichroism studies and microcomplement fixation techniques (unpublished results). No direct evidence has shown any substrate binding to the reduced enzyme. Indirect evidence that there is no binding is provided with nuclear magnetic resonance studies on the binding of a competitive anion, trifluoroacetate (Cheng and Martinez-Carrion, 1972), in which α-methylaspartate (K_d = 0.7 m*M*) easily displaced trifluoroacetate (K_d = 30 m*M*) from the native enzyme but not from the reduced enzyme. After reduction of the internal aldimine linkage, formation of the intermediate covalent intermediates is not possible. All covalent binding should be greatly reduced since the aldimine nitrogen carboxyl binding side is no longer available (Jenkins and D'Ari, 1966b).

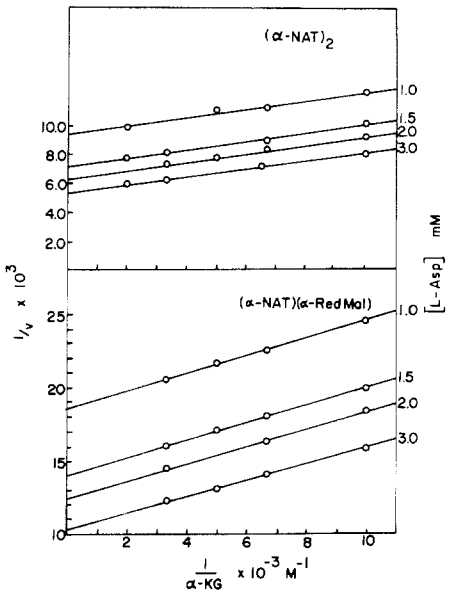

FIGURE 4: Double reciprocal plots of the steady-state kinetics measured in 0.05 *M* Tris-cacodylate (pH 8.3). Velocities are expressed as micromoles of oxalacetate formed per minute per milligram of enzyme.

Although the conditions for dissociating the enzyme are rather drastic, the native fraction, when compared with the control, retains nearly all its original specific activity (see Table I). The ratio of forms is not 1:2:1 but approaches this ratio if more drastic dissociating conditions are used at the expense of enzymatic activity and yield. The overall yield is not good (recovery of ~30% total protein and ~10% hybrid) but the method is rapid for preparation of large amounts of the hybrid with the preparation of 50 mg of hybrid not being too difficult.

The spectral properties confirm that the hybrid has half the amount of "actively bound" PLP as the native. Steady-state kinetics give values consistent for an enzyme which has two independent active sites since the V_{max} of the hybrid is the average of the native and reduced enzymes and the K_m values for both forms are the same. Throughout this work at the enzyme concentrations used to measure the spe-

cific activities and steady-state kinetics the enzyme remains a dimer. This has been demonstrated using sucrose density gradient centrifugation where the enzyme remains a dimer to the limits of detection (1×10^{-10} *M*) (Feliss and Martinez-Carrion, 1970). This is a concentration 100 times more dilute than that used to measure the specific activities. Also, the ratio of specific activities of the native to the hybrid enzyme remains the same even when measured in the presence of sucrose indicating that at least under conditions of substrate saturation the presence or absence of sucrose is irrelevant.

It has been suggested that glutamate aspartate transaminase might be a possible candidate as a flip-flop or negative cooperative enzyme in terms of coenzyme binding (Arrio-Dupont, 1972) and modification of a single tyrosine residue with nearly full loss of activity (Christen and Riordan, 1970). The tyrosine modification was later shown to be a secondary modification reaction (Birchmeier et al., 1973). Also, for both the mitochondrial (Churchich and Lee, 1974) and supernatant (Schlegel and Christen, 1974) isozymes, addition of 0.5 equiv of the coenzyme to the apoenzyme gives a mixture of species consistent with a random addition of the coenzyme as expected for a dimer with independent active sites. In addition, steady-state and transient kinetics as well as direct titrations with substrates and substrate analogs have not revealed any active site interactions (Braunstein, 1973). Nor has half-site conversion (which would be expected for a flip-flop mechanism) of pyridoxal phosphate to pyridoxamine phosphate or the reverse been observed (Jenkins and D'Ari, 1966a). Although there exists a very remote chance that $NaBH_4$ reduction destroyed any cooperative interactions it is very unlikely that the hybrid would then have half the activity of the native form. From the above evidence and the results of the present study, it is concluded that glutamate aspartate transaminase is not cooperative nor does it have a compulsory flip-flop mechanism for catalysis.

In general the active site hybrid method can be a valuable tool for looking at possible site–site interactions as has been shown here with glutamate aspartate transaminase where no detectable protein conformational alteration occurs during the hybridization process. In other systems where conflicting reports concerning site–site interactions have been reported the hybrid method could also prove useful if hybrids of active and inactive forms of the enzyme could be prepared.

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