Purification and Some Properties of Mouse Brain γ-Aminobutyric–α-Ketoglutaric Acid Transaminase*

A. Waksman and E. Roberts

ABSTRACT: γ-Aminobutyric–α-ketoglutaric acid transaminase was purified from mouse brain by a procedure utilizing extraction of acetone powder, ammonium sulfate precipitation, ammonium sulfate gradient elution, and calcium gel fractionation. A protein was obtained which appeared to be relatively homogeneous by carboxymethylcellulose column chromatography and gave a single major boundary in the ultracentrifuge. The optimum pH for the enzyme is 7.95. The K_m values for γ-aminobutyric acid and α-ketoglutaric acid were 1.2 × 10^{-2} and 2.0 × 10^{-3} M, respectively. The possibility is suggested that the strong association–dissociation tendency of the enzyme may be attributable to its tertiary structure.

For some time the work in our laboratory has been concerned with studies of the metabolic and physiological relationship of γ-aminobutyric acid (γ-ABA[1]), an easily extractable simple substance with a unique occurrence in the central nervous system of vertebrate organisms, in which it is found in extracts of brain, spinal cord, and retina. γ-ABA is formed to a large extent, if not entirely, from L-glutamic acid by an L-glutamate decarboxylase found in mammalian organisms only in the central nervous system. The first step in the metabolic utilization of γ-ABA, the reversible transamination of γ-ABA with α-ketoglutarate, is catalyzed by a transaminase which is found in the central nervous system and in other tissues. Both the decarboxylase and transaminase are B_6 enzymes. The products of the transaminase reaction are succinic semialdehyde and glutamic acid. Brain also contains a dehydrogenase which catalyzes the oxidation of succinic semialdehyde to succinic acid, which in turn can be oxidized *via* the reactions of the tricarboxylic acid cycle (see Roberts and Eidelberg, 1960, for many pertinent references). Hydroxylamine and aminooxyacetic acid, substances which produce marked elevations in γ-ABA content of brain of animals, were found to inhibit the γ-ABA–α-ketoglutaric acid transaminase activity *in vivo* without appreciably affecting the activity of glutamic acid decarboxylase (Baxter and Roberts, 1961). This contrasts with *in vitro* studies in which both enzymes were inhibited by these compounds at low concentrations. Since the γ-ABA system probably plays an important modulatory role in the activity of the central nervous system, it is necessary to learn more about the enzymes of γ-ABA metabolism. The present report deals with the purification of the γ-ABA–α-ketoglutaric acid transaminase, the first step in its characterization.

Experimental Procedure

Chemicals and Reagents. All reagents were obtained from commercial sources and were the purest grades available. The chemicals and sources were as follows: salts, acids, and bases, as well as the acetone were either Baker Analyzed reagent from J. T. Baker Chemical Co., Phillipsburg, N. J., or Mallinckrodt Analytical reagent from Mallinckrodt Chemical Works, St. Louis, Mo.; pyridoxal phosphate and α-ketoglutaric acid were Grade A, California Corp. for Biochemical Research, Los Angeles, Calif.; reduced glutathione, Schwarz Bio-Research, Inc., Orangeburg, N. Y.; Hyflo SuperCel (Celite), Fisher Scientific Co., Manufacturing Chemists, Fair Lawn, N. J.; γ-aminobutyric acid, General Mills, Inc., Minneapolis 13, Minn.; α-ketoglutaric-5-^{14}C (6.93 mcuries/mmole), International Chemical and Nuclear Corp., City of Industry, Calif., and Nuclear-Chicago Corp., Des Plaines, Ill.; carboxymethylcellulose, Bio-Rad Laboratories, Richmond, Calif.; reagents for scintillation counting, Packard Instrument Co., Inc., LaGrange, Ill.; and hydroxylamine HCl, Eastman Organic Chemicals, Rochester 3, N. Y.

Precautions against Inactivation. In previous studies it was demonstrated that γ-ABA–α-ketoglutaric acid transaminase is inhibited by substances which react with protein sulfhydryl groups or with pyridoxal phosphate (Baxter and Roberts, 1958). Therefore, most procedures employed included GSH and pyridoxal phosphate in the suspending medium. All steps were carried out in the cold (0–4°).

Preparation of the γ-Aminobutyric–α-Ketoglutaric Acid Transaminase

Preparation of an Acetone Powder. Mouse brain ace-

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[1] Abbreviations used in this work: γ-ABA, γ-aminobutyric acid.

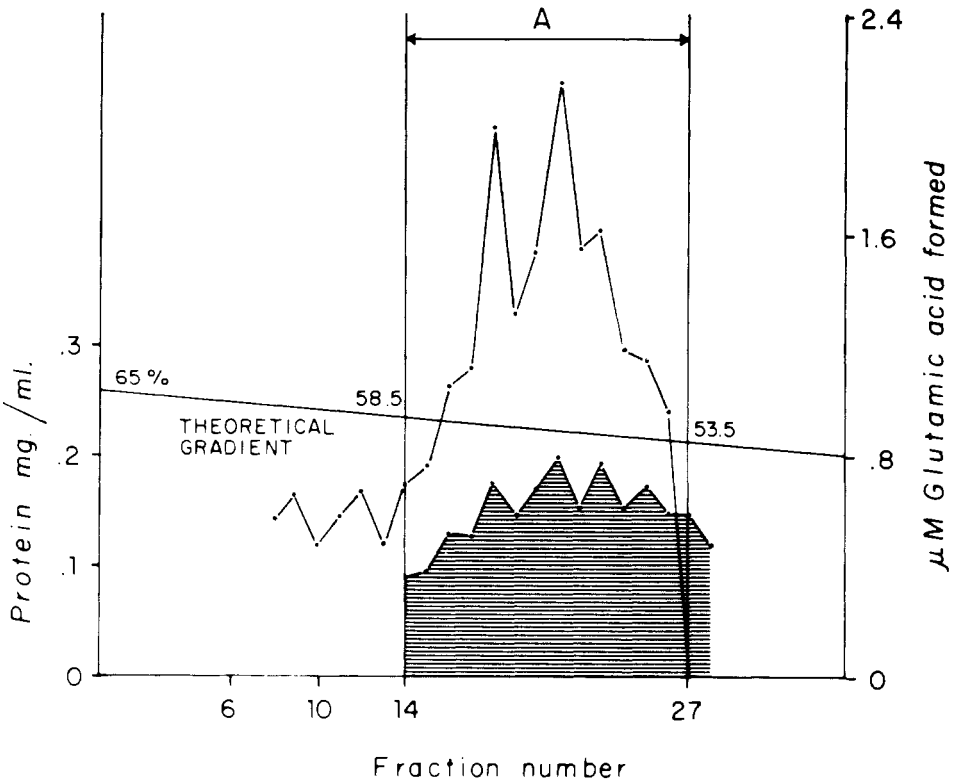

FIGURE 1: Ammonium sulfate gradient elution pattern of the γ-aminobutyric–α-ketoglutaric acid transaminase of mouse brain. The cross-hatched area under the lower curve shows the protein levels, while the area under the upper curve represents the enzyme activities of fractions 14–17 which were employed for further purification.

tone powder was used as a source of enzyme and was prepared as described previously (Roberts and Frankel, 1951). To prevent the loss of activity of the preparations, which was found to occur even on storage *in vacuo*, approximately 10 mg of GSH and 5 mg of pyridoxal phosphate were added to each 700-ml portion of acetone at −20° in a Waring blendor prior to suspending approximately 75 mouse brains therein. Mice were killed by cervical dislocation and whole mouse brains were dissected out rapidly and kept on ice in batches of approximately 25 until ready for use.

Extraction. Acetone powder (5 g, from approximately 100–200 mouse brains) was suspended with slow stirring in 50 ml of 0.01 M sodium phosphate buffer, pH 7.2. This suspension was stirred with a magnetic stirrer for 5 min and then homogenized in a large, chilled, all-glass homogenizer. After centrifugation of the suspension for 20 min at 15,000 × *g* at 4° in a Servall Model CR2 centrifuge and a twofold re-extraction with 30 ml of cold buffer, the combined supernatant fluid (approximately 100 ml) was stored at 0°.

Ammonium Sulfate Precipitation. Pulverized solid ammonium sulfate was added slowly with stirring at 0° over a period of 45 min to the above extract until 52.5% saturation was attained. After equilibration for 1 hr the precipitate was discarded and the saturation of ammonium sulfate raised to 60%. The precipitate was allowed to equilibrate for 1 hr and was removed by centrifugation for 15 min at 15,000 × *g*.

Ammonium Sulfate Gradient Elution. The above precipitate was suspended in 20 ml of phosphate buffer, pH 7.2, 65% saturated with ammonium sulfate. Celite was added to the suspension to form a light slurry. The slurry was poured onto a coarse sintered glass filter (5 cm in diameter) on top of a thin layer (5 mm) of wet Celite previously equilibrated with a 65% saturated solution of ammonium sulfate. After allowing the enzyme preparation to drain, an exponential gradient of ammonium sulfate was run through. The mixing chamber contained 125 ml of phosphate buffer solution (0.01 M, pH 7.2) brought to 65% saturation with ammonium sulfate. The funnel was filled with the same buffer saturated to the extent of 40% with ammonium sulfate (Schwimmer, 1953; Sayre and Hill, 1957). Fractions of 80 drops each were collected. A yellow peak containing the enzyme was eluted between 58.5 and 53.5% saturation with ammonium sulfate (Figure 1). The enzyme solution was dialyzed with stirring at 4° against three changes of 4-l. volumes of 0.01 M phosphate buffer, pH 7.2.

Calcium Phosphate Gel Fractionation. The dialyzed enzyme solution (about 100 ml) was then treated with 2 ml of calcium phosphate gel slurry (0.108 g dry weight/ml) (Tiselius *et al.*, 1956), stirred 30 min at 0°, and

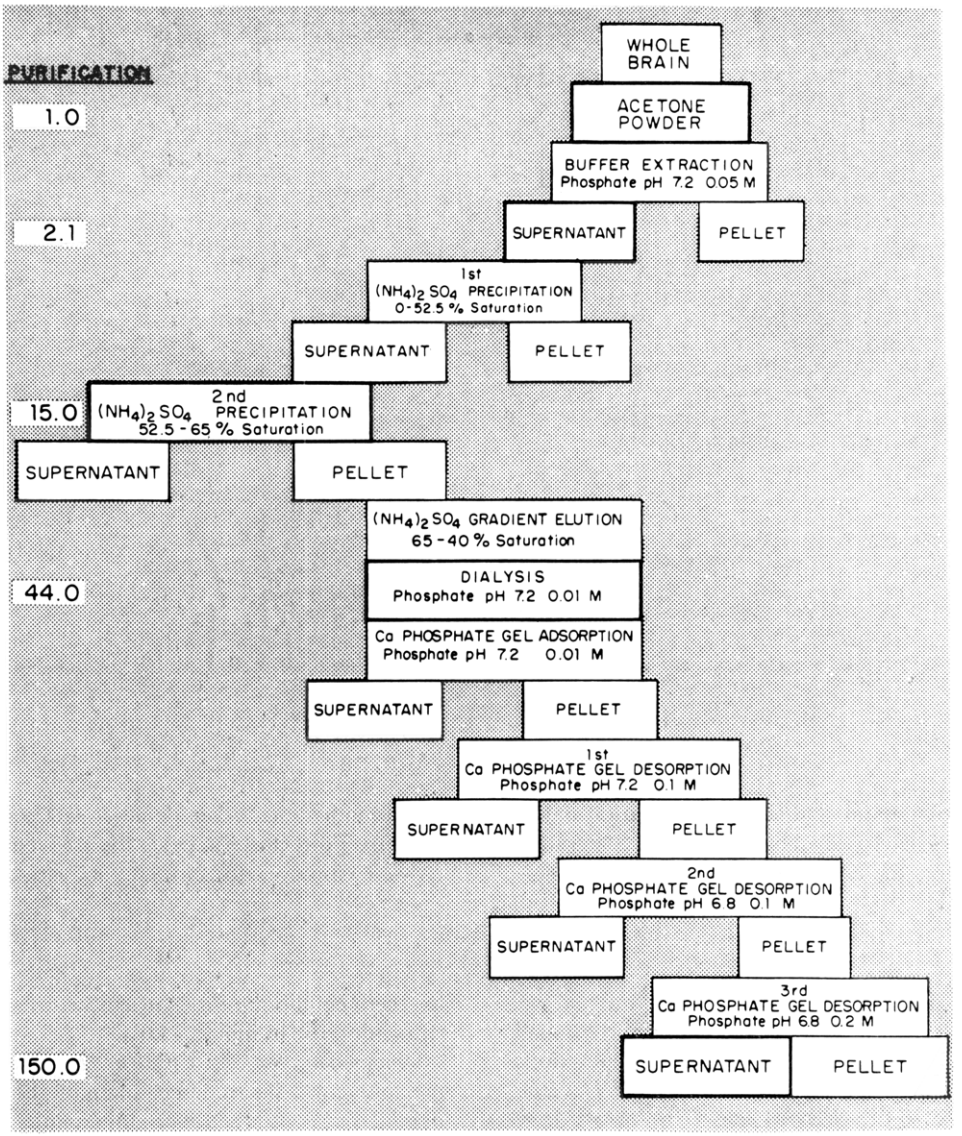

FIGURE 2: Purification flow diagram of mouse brain γ-aminobutyric–α-ketoglutaric acid transaminase.

centrifuged for 15 min at 20,000 × g at 4°. This treatment was repeated twice. The pellets were pooled and eluted with 10 ml of 0.1 M phosphate buffer, pH 7.2, for 10 min at 0° and the suspension was centrifuged for 10 min at 20,000 × g at 4°. The pellet was resuspended in 10 ml of 0.1 M phosphate buffer, pH 6.8, and treated as above. The final pellet was eluted with 3 ml of 0.2 M phosphate buffer, pH 6.8, for 30 min and then centrifuged. The latter step was repeated twice with 2 ml of the buffer. The supernatant fluid from the latter treatment containing γ-ABA–α-ketoglutaric acid transaminase (usually approximately 150-fold purified) was pooled and dialyzed overnight against 4 l. of phosphate buffer, pH 7.2, 0.01 M. The dialyzed preparation was then stored under nitrogen in the dark at 4° and usually was found to be stable for at least three days.

Assays

Principle. The formation of glutamic acid in the following transamination reaction has been used to measure the enzymatic activity:

$$\gamma\text{-aminobutyrate} + \alpha\text{-ketoglutarate} \rightleftarrows \text{succinic semialdehyde} + \text{glutamate}$$

Glutamic Acid Determinations. Glutamic acid was determined according to the methods of Baxter and Roberts (1958) or of Waksman and Roberts (1963).

Enzyme Assay. For assay of enzyme solutions incubations were performed for 1 hr at 37° in capped, round-bottom centrifuge tubes or in 10-ml erlenmeyer flasks in a Dubnoff incubator in a final volume of 1.5

TABLE I: Steps in the Purification of Mouse Brain γ-Aminobutyric–α-Ketoglutaric Acid Transaminase.

Fractions	Total Activity (%)	Total Protein (%)	Specific Activity[a]	Purification
Acetone powder	100	100.0	0.21	1.0
Extract	100	46.0	0.43	2.1
$(NH_4)_2SO_4$ precipitate	27	3.0	3.2	15.0
$(NH_4)_2SO_4$ gradient eluate	14	0.9	8.8	44.0
Calcium phosphate gel fraction	12[b]	0.4	30.0[b]	150.0

[a] Micromoles of glutamate formed/hour mg^{-1} of protein under the standard conditions of assay. [b] Corrected for optimal concentration of enzyme.

ml containing the following substances: 0.2 ml or 0.4 ml enzyme solution; 50 μM each of α-ketoglutarate and γ-aminobutyrate; 100 μM of pH 8.2 borate buffer; and 15 μg each of pyridoxal phosphate and neutralized reduced glutathione. The final pH of the incubation mixture was 7.95. The reaction was stopped by addition of 0.5 ml of 25% trichloroacetic acid and the glutamic acid was determined. Comparable low blanks obtained either on incubation of substrate with boiled enzyme or when enzyme was omitted were subtracted. The method of Waksman and Roberts (1963) was also used, with comparable results.

Protein Determination. Protein determinations were made by the procedure of Lowry *et al.* (1951).

Carboxymethylcellulose Column Chromatography. Carboxymethylcellulose column chromatography was performed on purified enzyme preparations. A 13.4-ml sample, containing about 0.5 mg of protein/ml, was adjusted to 0.1 M phosphate buffer at pH 6.7 and poured onto a carboxymethylcellulose column (50 × 1.4 cm) previously equilibrated with 0.1 M phosphate buffer at pH 6.7. The elution pattern of the enzyme, obtained with 0.1 M phosphate buffer at pH 6.7, was followed. Fractions of 60 drops (2.1 ml) were collected. γ-ABA–α-ketoglutaric acid transaminase determinations were made according to the method of Waksman and Roberts (1963) on 0.1-ml aliquots of the eluted fractions. Protein determinations were made on the same fractions.

Results

Purification. A typical result of the purification procedure described above is summarized in Figure 2 and in Table I showing a 150-fold increase in the specific activity of the purified enzyme over the acetone powder. The purified enzyme gave an average activity of 30 μmoles of glutamate formed/hour mg^{-1} of protein. Approximately the same degree of purification was obtained in a number of runs.

Carboxymethylcellulose Column Chromatography. A typical elution pattern of γ-ABA–α-ketoglutaric acid transaminase activity and protein emerging from a carboxymethylcellulose column is shown in Figure 3.

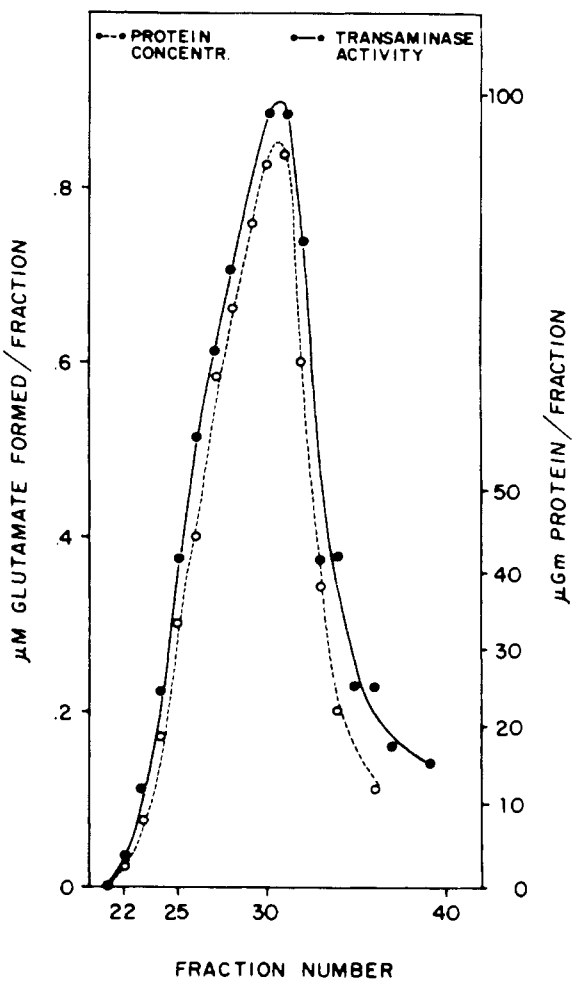

FIGURE 3: Chromatography on carboxymethylcellulose of purified γ-aminobutyric–α-ketoglutaric acid transaminase. The column, 50 × 1.4 cm, was eluted with 0.1 M phosphate buffer at pH 6.7.

The results in the example show that no further purification of the enzyme could be achieved by this method and that 84.5% of the total protein and 87.5% of the total enzymatic activity were recovered. The results suggest

the presence of one major component in our preparation.

Ultracentrifugation. The Schlieren patterns obtained with 30-mm double-sector synthetic-boundary, capillary-type cells, using enzyme preparations containing respectively 1, 2, 3.5, and 7 mg of protein/ml, buffered at pH 7.2 (0.01 M phosphate), show one single major boundary. An example is shown in Figure 4. The approximately (±5%) constant area under the peaks would also show the absence of polydisperse, rapidly moving material contaminating the main constituent. The calculated $s_{20,w}$ value extrapolated to time 0 and at infinite dilution is 5.0 (Figures 5A and 5B), if it is assumed that there is no change in the state of aggregation of the enzyme at low protein concentration. If a globular shape is assumed, this value would be given by a protein with a molecular weight of approximately 100,000. The slight deformation of the peak at the end of the ultracentrifugal run and the observed spreading of the boundary is compatible with an equilibrating system (Reithel, 1963). This hypothesis is consistent with the finding that the specific activity of the enzyme undergoes drastic changes with increasing enzyme concentration. The curve passes through a maximum, suggesting the possibility of the existence of at least three different forms of the enzyme. The ultracentrifugation runs of concentrated enzyme preparations (3.5–7 mg/ml) performed either in the presence of 1% Tween 80 or in a mixture containing 0.5 M α-ketoglutarate and 0.5 M glutamate gave calculated $s_{20,w}$ values, respectively, of 1.7 and 22.1, assuming no preferential adsorption on the enzyme of the added substances. The three values for $s_{20,w}$ (1.7, 5.1, and 22.1) obtained under the various conditions of ultracentrifugation are in agreement with the probable existence of at least three different active forms of the enzyme in association–dissociation equilibria. This phenomenon is presently under study in this laboratory.

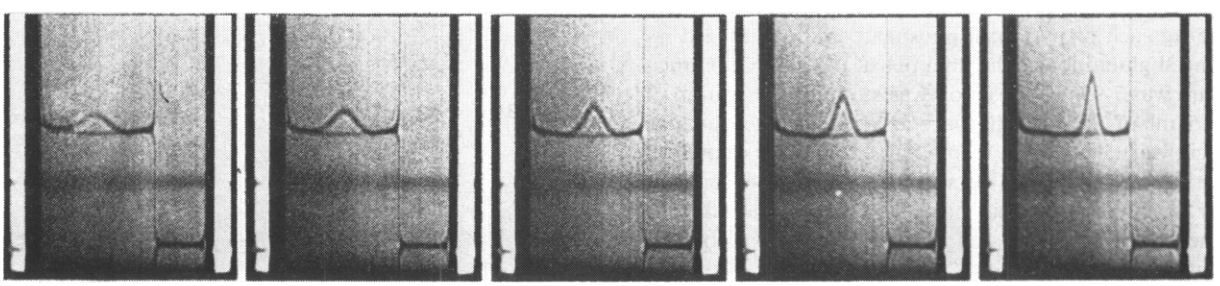

FIGURE 4: Schlieren pattern from purified γ-aminobutyric–α-ketoglutaric acid transaminase obtained in a 30-mm double-sector, synthetic-boundary, capillary-type cell. The speed used was 50,740 rpm. The first picture was taken when this speed was reached. The interval between the pictures was 8 min. Schlieren angle used was 50°. The gravitational field is from right to left. The calculated $s_{20,w}$ is 5.0 (extrapolated).

Effect of Hydroxylamine on Sedimentation Coefficient. One could speculate that the reason for the very strong protein–protein interaction existing in our enzyme preparation might be, at least in part, attributable to the pyridoxal phosphate moiety of the enzyme. The data in the literature could be consistent with the interpretation that the aldehyde group of pyridoxal phosphate can be attached in thiazolidino linkage to closely lying cysteine sulfhydryl and lysine ε-amino groups or that an imidazolidino linkage can occur between the aldehyde group and two properly disposed lysine ε-amino groups (Roberts *et al.*, 1964). It can thus be envisioned that the pyridine ring of the coenzyme is attached to one portion of a protein and the aldehyde group to another segment of another molecule of the same protein, thus building up the observed aggregates. This hypothesis was tested by measuring the sedimentation coefficient of our enzyme preparations after treatment with 10^{-1} and 10^{-3} M hydroxylamine, respectively. Under similar conditions 10^{-3} M hydroxylamine inhibits the enzyme activity completely. Enzyme solutions (1 ml each) containing about 1 mg of protein/ml were treated at 0° for 30 min with the above concentration of hydroxylamine and dialyzed for 3 hr at 4° against 2 l. of 0.01 M phosphate buffer, pH 7.2, containing the same concentration of hydroxylamine as the samples to be dialyzed. The sedimentation coefficients were measured in 30-mm double-sector synthetic-boundary, capillary-type cells. The $s_{20,w}$ calculated nonextrapolated values were 5.3 and 5.8, respectively, in 10^{-1} and 10^{-3} M hydroxylamine. These values were essentially the same as those obtained with untreated enzyme (5.2 nonextrapolated value) and indicate that hydroxylamine, unlike Tween 80, does not dissociate γ-ABA–α-ketoglutaric transaminase. Since under the conditions employed it may be presumed that the aldehyde group of the coenzyme is completely bound by hydroxylamine, the results indicate that the aldehyde group of the pyridoxal phosphate cofactor might not be involved in the aggregation of the enzyme.

Effect of pH on the Activity of the γ-ABA–α-Ketoglutaric Acid Transaminase. A typical curve of γ-ABA–α-ketoglutaric transaminase activity as a function of pH is shown in Figure 6. The results in the example show the pH optimum of the enzyme to be between pH 7.95 and 8.00. Potassium phosphate buffers (0.2 M) were used for the incubation mixtures at a final pH of 6.85,

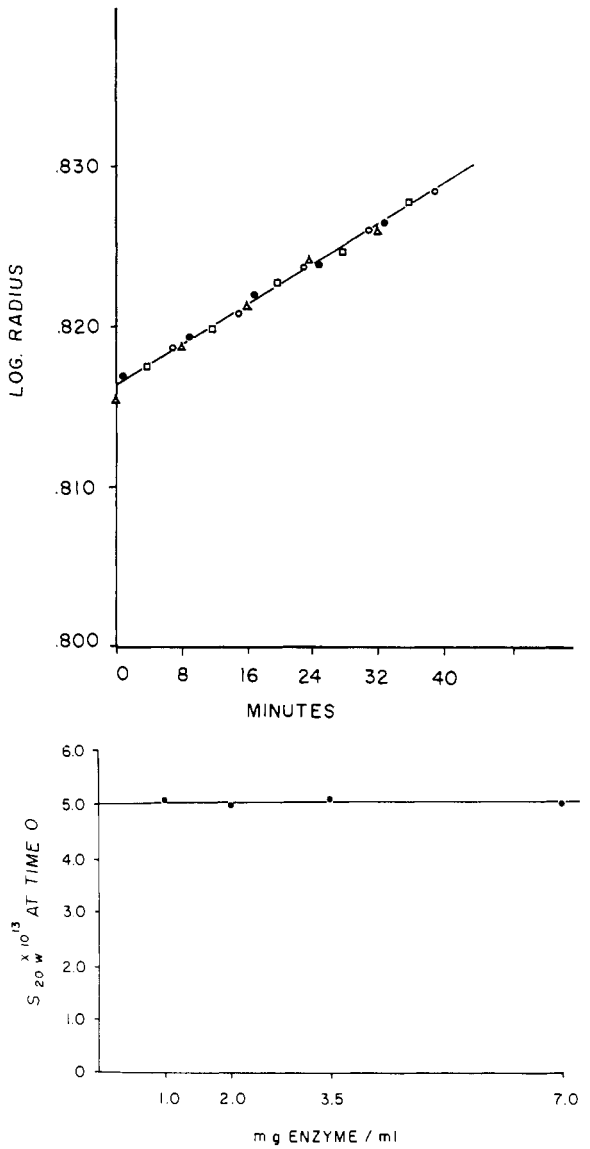

FIGURE 5: Determination of sedimentation coefficient of the enzyme. (A) From movement of boundary. Concentration of protein used in the runs: Δ—Δ, 1 mg/ml; □—□, 2 mg/ml; O—O, 3.5 mg/ml, and ●—●, 7 mg/ml. Abscissa: time after reaching maximum speed. The parallel lines obtained from the separate runs at different protein concentration were transposed graphically at the level of the 1 mg/ml curve. (B) Sedimentation coefficient of the enzyme as a function of the protein concentration.

7.00, 7.25, 7.80, and 7.95, sodium borate buffers (0.2 M) were used for the incubation mixtures at a final pH of 7.50, 7.65, 7.85, 8.20, 8.40, 8.60, 8.80, and 8.90. Enzyme activity was measured according to Waksman and Roberts (1963); 0.1 mg of dialyzed enzyme/incubation was used. The enzyme had been dialyzed against 500 volumes of phosphate buffer at pH 7.2, 0.01 M.

Michaelis–Menten Constants of γ-ABA–α-Ketoglutaric Acid Transaminase. The K_m values for α-ketoglutaric acid and for γ-ABA (Figure 7) were 2.0×10^{-3} and 1.2×10^{-2} M, respectively, as calculated from the Lineweaver–Burk plots.

Effect of Enzyme Concentration. The specific activity of the γ-ABA–α-ketoglutaric acid transaminase was determined at varying enzyme concentrations. A typical specific activity curve is shown in Figure 8. It shows a maximum at an enzyme concentration of 0.1 mg/ml of incubation mixture, suggesting the existence of at least three possible aggregate forms of the enzyme. This possibility is also supported by the ultracentrifugal data.

Discussion

The procedure presented here for the isolation and purification of γ-ABA–α-ketoglutaric acid transaminase from mouse brain has been repeated several times with similar results. It yielded a preparation of enzyme 150-fold purified by comparison with the initial acetone powder extracts.

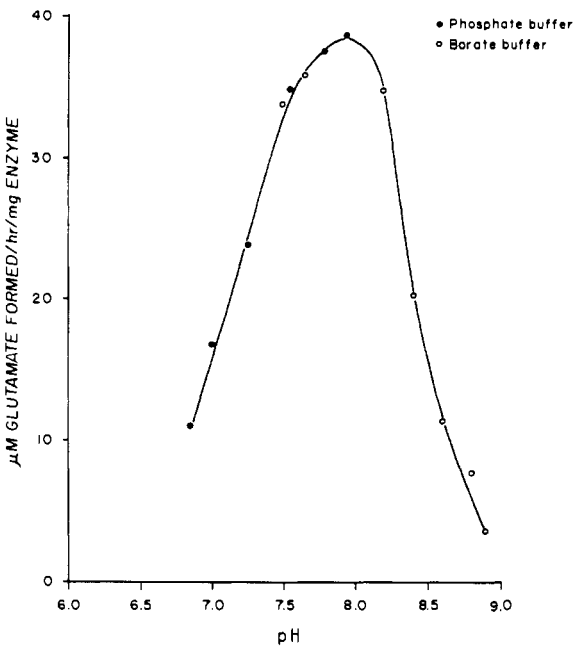

FIGURE 6: Specific activity of the enzyme as a function of the pH.

The protein showed a very strong tendency for aggregation, which was indicated by the changes in specific activity of the enzyme as a function of its concentration. This was also shown by the variations of the ultracentrifugal patterns in the presence of Tween 80 or when contained in a mixture of α-ketoglutarate and glutamate. Enolase from ox brain also has been found to show a very strong tendency for association (Wood, 1964).

The ultracentrifugal behavior of γ-ABA–α-keto-

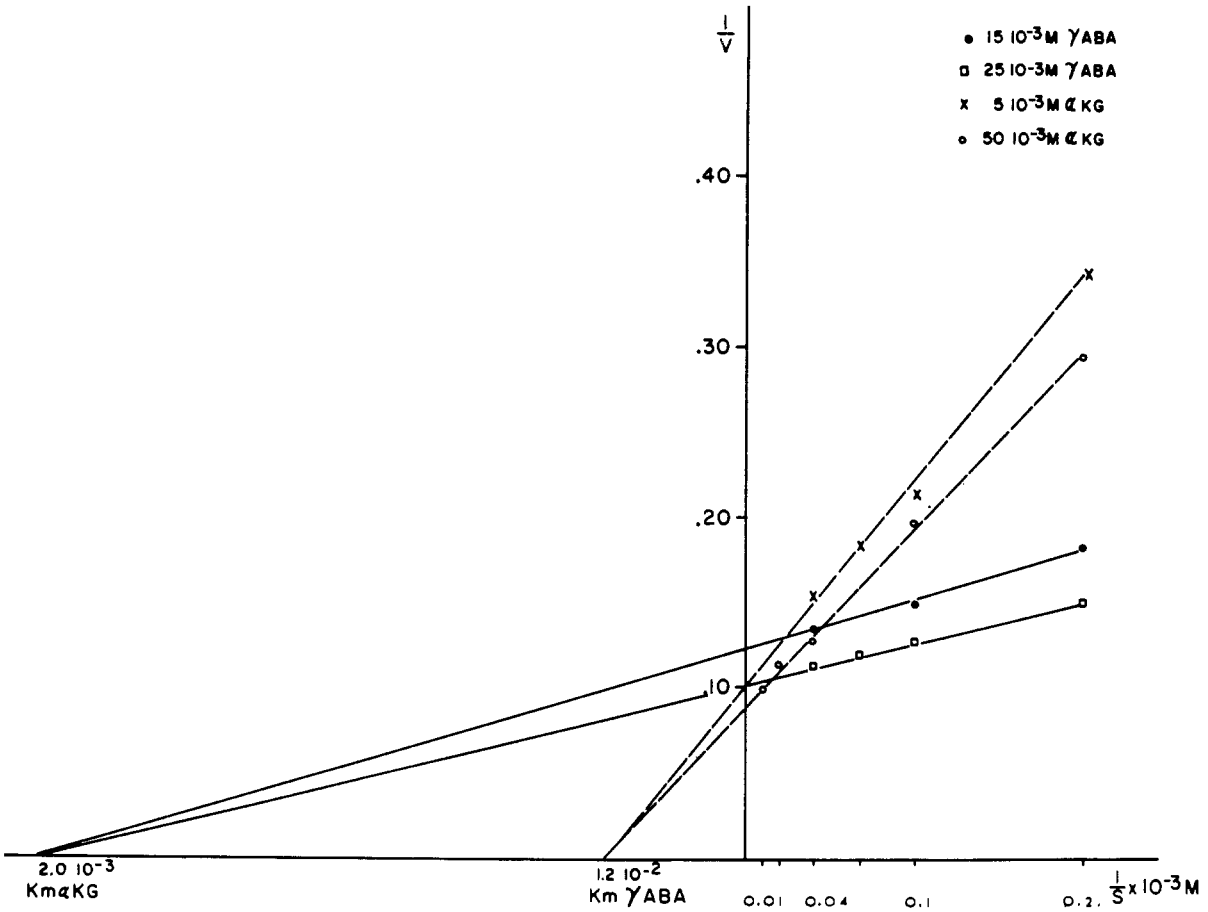

FIGURE 7: K_m values for α-ketoglutaric acid enzyme and γ-ABA-enzyme complexes. Lineweaver–Burk plots. $1/V$ = 1 μmole of glutamate formed/hr mg^{-1} of protein.

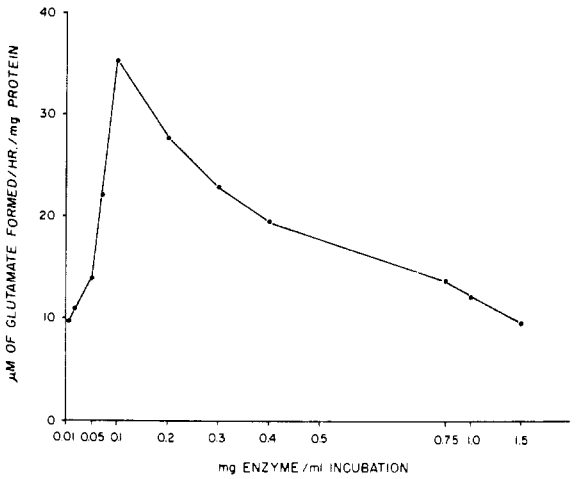

FIGURE 8: Specific activity of the enzyme as a function of the protein concentration.

glutaric acid transaminase in Tween 80 and in hydroxylamine as compared to untreated enzyme suggests that the tertiary structure of the protein rather than the aldehyde group of the coenzyme might be the prevailing factor in the aggregation phenomenon observed in our enzyme preparation.

Acknowledgments

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Photooxidation with Rose Bengal of a Critical Histidine Residue in Yeast Enolase*

Edward W. Westhead

ABSTRACT: Rose bengal has been used as a photoactivated oxidant to inactivate yeast enolase. The pH dependence of the rate of inactivation shows that oxidation of a histidine group is responsible for the loss of activity, and since the inactivation is first order to less than 0.1% residual activity either a single group or two or more exactly equivalent groups are involved. Amino acid analyses and other data support this interpretation but also show that oxidation of other, noncritical, amino acids also occurs. The enzyme is partially protected by Mg^{2+} and maximally protected by Mg^{2+} and substrate. Substrate alone does not protect. Rose bengal was found to be far more specific and less generally damaging to the enzyme than methylene blue.

The description of the active site of any enzyme is interesting as a contribution to our knowledge of chemical reaction mechanisms. In addition we may hope that the accumulation of such descriptions for a variety of enzymes with diverse catalytic properties may show a pattern of mechanism that will be interesting in terms of biological development. Although direct chemical evidence for the participation of a specific group in catalysis is most convincing, this approach suffers from a lack of reagents specific for "active site" groups. Weil *et al.* (1953, 1955) have shown that for some enzymes photooxidation with methylene blue is sufficiently specific to implicate a histidine residue in the catalytic mechanism. This reagent has since been used successfully by others (*e.g.*, Ray *et al.*, 1960), but more frequently investigators have found that so many groups were oxidized at similar rates that no sure interpretation could be placed on the results.

The present work was initiated in the hope that replacement of the cationic methylene blue by an anionic photoactive dye would lead to greater specificity for the active sites of enzymes that bind anionic substrates. Rose bengal was chosen on the basis of studies by Oster *et al.* (1959) which describe the photooxidizing efficiencies of a large number of dyes. Brake and Wold (1960) had previously described the photooxidation of yeast enolase by methylene blue and concluded that the reagent lacked sufficient specificity to allow assignment of a critical role to any particular residue. The present work therefore provides a clear test of the relative usefulness of rose bengal in this particular case.

Experimental Section

Materials. Rose bengal was obtained from the Fisher Scientific Co. and was manufactured by the National Aniline Division of Allied Chemical Corp. Although it was found to contain several components by chromatography on alumina (Jirsa and Raban, 1962), the dye was used without purification except for passage through a column of Dowex 50 resin in the sodium form to remove other cations. This dye is a highly halogenated fluorescein dye and in its pure form is tetraiodotetrachlorofluorescein. Amino acids and their derivatives were the best grades obtainable from Calbiochem. Enolase was prepared and assayed as previously described (Westhead and McLain, 1964); chromatography on TEAE-cellulose was carried out as described in that paper except that it was done at room temperature rather than at 4°. D-Phospholactic acid samples were gifts from Drs. Clinton Ballou and Finn Wold.

Methods. Amino acid analysis was done with a Spinco Model 120 analyzer. Samples were hydrolyzed in 6 N hydrochloric acid at 110° for 25 and 40 hr in an atmosphere of oxygen-free argon. The values for the leucine, arginine, and phenylalanine content of enolase were found to be very reproducible and were routinely used as standards in calculating μmoles of other amino acids $mole^{-1}$ of enzyme when small amounts of protein were hydrolyzed.

Oxidation was followed using a Clark polarographic

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