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Chicken Heart Soluble Aspartate Aminotransferase. Purification and Properties*

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ABSTRACT: Chicken heart soluble aspartate aminotransferase has been purified, and both the holo- and apoenzymes have been crystallized. Pyridoxal phosphate stabilizes the apoenzyme against denaturation by heat, urea, and extremes of pH. The binding of the coenzyme to the apoenzyme decreases the fluorescence of the tryptophan residues in the enzyme and increases the protein polarization of fluorescence.

The minimum weight of enzyme per mole of bound coenzyme has been determined by fluorescence titrations to be 52,000 ± 5000 for pyridoxal phosphate and 48,900 ± 4000 for pyridoxamine phosphate and by polarization of fluorescence measurements to be 48,700 ± 4000 for pyridoxal phosphate.

The native enzyme has a sedimentation coefficient of 5.5 and a sedimentation equilibrium molecular weight of 100,000. This 5.5S enzyme is dissociated at pH 3.0 into a 2.45S component. Sedimentation equilibrium ultracentrifugation of dilute holoenzyme solutions at pH 9 and 10 demonstrates an equilibrium between the monomer and dimer. An enzymatically active tetramer (8.1 S; molecular weight, 190,000) has been isolated which can be dissociated in 1×10^{-2} M 2-mercaptoethanol to the 5.5S protein.

The existence of two electrophoretically different forms of aspartate aminotransferase (L-aspartate:2-oxoglutarate aminotransferase (2.6.1.1) were observed in dog heart extracts by Fleisher (1960). Differential centrifugation of rat liver extracts, also containing these two forms, demonstrated that the anionic protein was associated with the supernatant fraction of the cell, while the cationic form was associated with the mitochondria (Boyd, 1961, 1962).

The mitochondrial and soluble forms of the aspartate aminotransferase have been purified and characterized from a number of mammalian tissues: beef heart (Morino *et al.*, 1963), pig heart (Nisselbaum and Bodansky, 1966; Martinez-Carrion and Tiemeier, 1967), rat liver (Hook and Vestling, 1962; Harpring, 1965), and human tissues (Bodansky *et al.*, 1966).

We have observed that both the anionic and cationic forms of aspartate aminotransferase are also present in chicken heart extracts. This paper reports the purification and crystallization of the soluble, anionic form of chicken heart AAT[1] and presents an investigation of the

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[1] Abbreviations used: AAT, aspartate aminotransferase; DPNH, reduced diphosphopyridine nucleotide.

fluorescence and molecular weight properties of the enzyme.

Materials

Chicken hearts were obtained from a commercial slaughterhouse. L-Aspartic acid and pyridoxal phosphate were purchased from Mann Research Corp., malic dehydrogenase and α-ketoglutaric acid from the California Corp. for Biochemical Research, pyridoxal and pyridoxamine from Sigma Chemical Co., DPNH from P & L Biochemicals, and bovine serum albumin from Armour Pharmaceutical Co. Sephadex G-100 was obtained from Pharmacia Fine Chemicals; DE-11 and CM-11 ion-exchange resins from Whatman. Urea was recrystallized from methanol before use.

Methods

Enzyme assays were performed by a modification of the method of Karmen (1955). All reagents were prepared in 0.1 M Tris-HCl buffer (pH 7.4) and the pH of L-aspartate and α-ketoglutarate solutions was adjusted to pH 7.4 with 10 M KOH. The total volume of the reaction mixture was 3.1 ml. The components of the reaction mixture were added in the following order: 1–2 μg of AAT, 0.27 μmole of pyridoxal phosphate, 3 μg of malic dehydrogenase, 0.29 μmole of DPNH, and 100 μmoles of L-aspartate. In order to ensure maximum binding of the coenzyme, all components of the assay mixture were incubated at room temperature for 20 min prior to the start of the assay. The reaction was initiated by the addition of 20 μmoles of α-ketoglutarate. The decrease in optical density at 340 mμ was recorded for 2 min. A unit of activity is expressed as $0.12 \times \Delta OD/15 \text{ sec} \times D \times 10^3$, where

$$D = \frac{1}{\text{ml of enzyme in cuvet}} \times \text{prior dilution of sample}$$

Specific activity is expressed as the ratio of units to the optical density at 280 mμ.

Starch Gel Electrophoresis. Horizontal starch gel electrophoresis was carried out in the apparatus described by Fine and Costello (1963). Gels were prepared in 0.03 M Tris-phosphate buffer (pH 8.5); the electrode buffer was 0.3 M Tris-phosphate (pH 8.5). The starch concentration was 14 g/100 ml of buffer. Gels were run with a current of 10 ma/gel for 17 hr at 5–10°. The gels were stained for enzymatic activity by the procedure of Decker and Rau (1963). The gels were stained for protein with 1% Amido Black.

Spectra. Absorption spectra were measured on a Cary 14 recording spectrophotometer. Fluorescence measurements were made with a Turner Model 201 recording spectrofluorometer. Polarization of fluorescence was measured with an Amico-Bowman spectrofluorometer fitted with Glan Thompson polarizers.

Ultracentrifugation. Sedimentation velocity runs were carried out in a Spinco Model E ultracentrifuge equipped with a schlieren optical system. All runs were at a speed of 59,780 rpm and at 22–25°. The viscosity of the buffer was assumed to be the same as that of water, and the observed sedimentation coefficient was corrected to the viscosity of water at 20°.

Sedimentation equilibrium runs were carried out in a Spinco Model E ultracentrifuge equipped with Rayleigh interference optics. Standard double-sector cells with quartz windows were used, and photographs were taken on Kodak spectrographic plates, emulsion type II-G. All runs were carried out for 18 hr, and in every run equilibrium conditions were established within this time period. Calculations were carried out according to the method of Yphantis (1964). For all runs, the plot of the natural logarithm of the y displacement *vs.* the x coordinate was linear. For both sedimentation velocity and sedimentation equilibrium calculations, the value of 0.74 was assumed for the partial specific volume of the enzyme.

Enzyme Purification. All ammonium sulfate fractionation steps were carried out by the addition of solid ammonium sulfate; the per cent saturation stated is the per cent saturation at 25°. All steps except the column chromatography were at 4°; columns were run at 22–25°. All dialyses were against six to ten volumes of buffer for a period of 8–12 hr. A summary of the purification procedure is given in Table I.

STEP 1. Frozen chicken hearts were ground in a meat grinder and extracted for 1 hr with 0.56 l. of distilled water/lb of hearts. The extract was filtered through cheesecloth and centrifuged for 30 min at 1300*g*. The supernatant was then filtered through glass wool, and the filtrate was brought to 70% $(NH_4)_2SO_4$. The 70% precipitate was removed by filtration, dissolved in cold distilled water, and dialyzed against distilled water. The 70% supernatant was brought to 90% $(NH_4)_2SO_4$, and the precipitate was removed by filtration. This 70–90% precipitate contains mainly the mitochondrial form of the enzyme.

STEP 2. The 0–70% precipitate was suspended in water and dialyzed against distilled water. The dialyzed extract was brought to 43% $(NH_4)_2SO_4$; the precipitate was removed by filtration and discarded. The 43% supernatant was brought to 80%; the precipitate was taken up in 0.05 M potassium phosphate (pH 6.5)–0.001 M EDTA and dialyzed against this buffer.

STEP 3. The enzyme solution was brought to 0.002 M in α-ketoglutarate (pH 7.4). The enzyme was heated to 55° in a 65° water bath and maintained at this temperature for 5 min. The heated solution was cooled in an ice bath to 10°, and the precipitate was removed by centrifugation.

STEP 4. The supernatant was brought to 60% $(NH_4)_2SO_4$. After centrifugation, the precipitate was dissolved in 0.005 M potassium phosphate buffer (pH 6.5)–0.001 M EDTA and dialyzed against this buffer.

STEP 5. The dialyzed enzyme was then applied to a CM-11 column which had previously been equilibrated with 0.005 M potassium phosphate buffer (pH 6.5)–0.001 M EDTA. Protein was applied (4 mg/ml of

TABLE I: Purification of Chicken Heart Soluble Aspartate Aminotransferase.[a]

Step	Total Protein (mg)	Total Act. (units)	Sp Act. (units/OD_{280})	Total Yield (%)
1. Crude extract	91.5×10^4	31.59×10^6	35	
2. 43–80% ammonium sulfate	38.4×10^3	9.65×10^6	254	30.4
3. Heat, 55°, 5 min	17.4×10^3	7.67×10^6	438	24.3
4. 0–60% ammonium sulfate	10.4×10^3	5.25×10^6	502	16.6
5. Chromatography on CM-11	8.8×10^3	5.05×10^6	570	15.9
6. Chromatography on DE-11	0.57×10^3	2.60×10^6	4540	8.4
7. Chromatography on Sephadex G-100	0.35×10^3	1.92×10^6	5500	6.1
8. Crystallization	0.29×10^3	1.82×10^6	6250	5.7

[a] Summary of the purification steps in the preparation of aspartate aminotransferase from 24 kg of chicken hearts as described in the text.

packed CM-11). After application of the enzyme, the column was washed with 0.005 M potassium phosphate buffer (pH 6.5)–0.001 M EDTA. The soluble AAT was eluted immediately from the column. The mitochondrial AAT was bound to the column under these conditions and could be eluted by a gradient of 0.005 M potassium phosphate (pH 6.5)–0.001 M EDTA in the mixing flask and 0.1 M potassium phosphate buffer (pH 6.5)–0.001 M EDTA in the reservoir flask. The volume of buffer in each flask was two to five times the column volume.

STEP 6. The soluble AAT fractions of highest specific activity were pooled and brought to 80% $(NH_4)_2SO_4$. After centrifugation, the precipitate was taken up in a small volume of 0.005 M potassium phosphate buffer (pH 7.5) and dialyzed against this buffer. The dialyzed enzyme was applied to a DE-11 column which had previously been equilibrated with 0.005 M potassium phosphate buffer (pH 7.5)–0.001 M EDTA. Protein was applied (4 mg/ml of packed DE-11). After application of the enzyme, the column was washed with 0.005 M potassium phosphate buffer (pH 7.5)–0.001 M EDTA. The soluble AAT was eluted with the initial wash and fractions of highest specific activity were pooled and brought to 80% $(NH_4)_2SO_4$. After standing for 8 hr, the precipitate was collected by centrifugation, taken up in a minimal volume of 0.05 M potassium phosphate buffer (pH 7.5), and dialyzed against this buffer.

STEP 7. After dialysis, the enzyme solution was applied to a Sephadex G-100 column which was equilibrated with 0.05 M Tris-HCl (pH 7.5)–0.1 M KCl. The height:diameter ratio of the column was 15:1; and the column volume was 10–20 times greater than the volume of the enzyme solution. The column was eluted with 0.05 M Tris-HCl (pH 7.5)–0.1 M KCl.

STEP 8. The fractions of highest specific activity were pooled and brought to 80% $(NH_4)_2SO_4$. The precipitate was collected by centrifugation at 20,000*g* for 30 min and dissolved in sufficient 0.05 M potassium phosphate buffer (pH 7.5) to give a protein concentration of 5–10 mg/ml. This solution was dialyzed against 0.05 M potassium phosphate (pH 7.5) and clarified by centrifugation. Finely powdered ammonium sulfate was slowly added until a faint turbidity appeared (about 50% saturation). During the addition of the ammonium sulfate, the pH was maintained at 7.0–7.5 (pH paper) by the addition of 2 N ammonium hydroxide. The enzyme solution was clarified by centrifugation at 20,000*g* for 30 min and left at 4°. Crystals appeared within 1–2 days. Both the holo and apo chicken heart soluble AAT's can be crystallized by this method. Figure 1 shows the holo-S-AAT crystals. The apoenzyme crystals are identical in appearance. Both the holo- and apoenzymes are stable for several months when stored at 4° as a suspension of crystals in 80% ammonium sulfate.

Both the holo- and apoenzymes prepared by this procedure have a specific activity of 6250 ± 250. The crystalline holoenzyme has a 280:260 ratio of 1.6; the crystalline apoenzyme has a 280:260 ratio of 1.8.

This purification procedure yields the apoenzyme if no pyridoxal phosphate is added during the preparation of the enzyme. A slightly larger yield of enzyme is obtained if sufficient pyridoxal phosphate is added during the course of purification to maintain the enzyme in the holoenzyme form.

Results

Purity. The enzyme prepared by the procedure given above was homogeneous in the ultracentrifuge at pH 7.5 in 0.05 M potassium phosphate buffer and at pH 9.0 in 0.05 M glycine–KOH buffer. Rabbit antibodies prepared with crystalline holoenzyme gave only one precipitating band when tested with either the crystalline enzyme or with crude chicken heart extract by double diffusion in agar according to the procedure of Stollar and Levine (1963).

Starch Gel Electrophoresis. It has previously been observed that starch gel electrophoresis of mammalian enzymes separates both the mitochondrial and soluble enzymes into a series of enzymatically active proteins (Decker and Rau, 1963; Martinez-Carrion *et al.*, 1967;

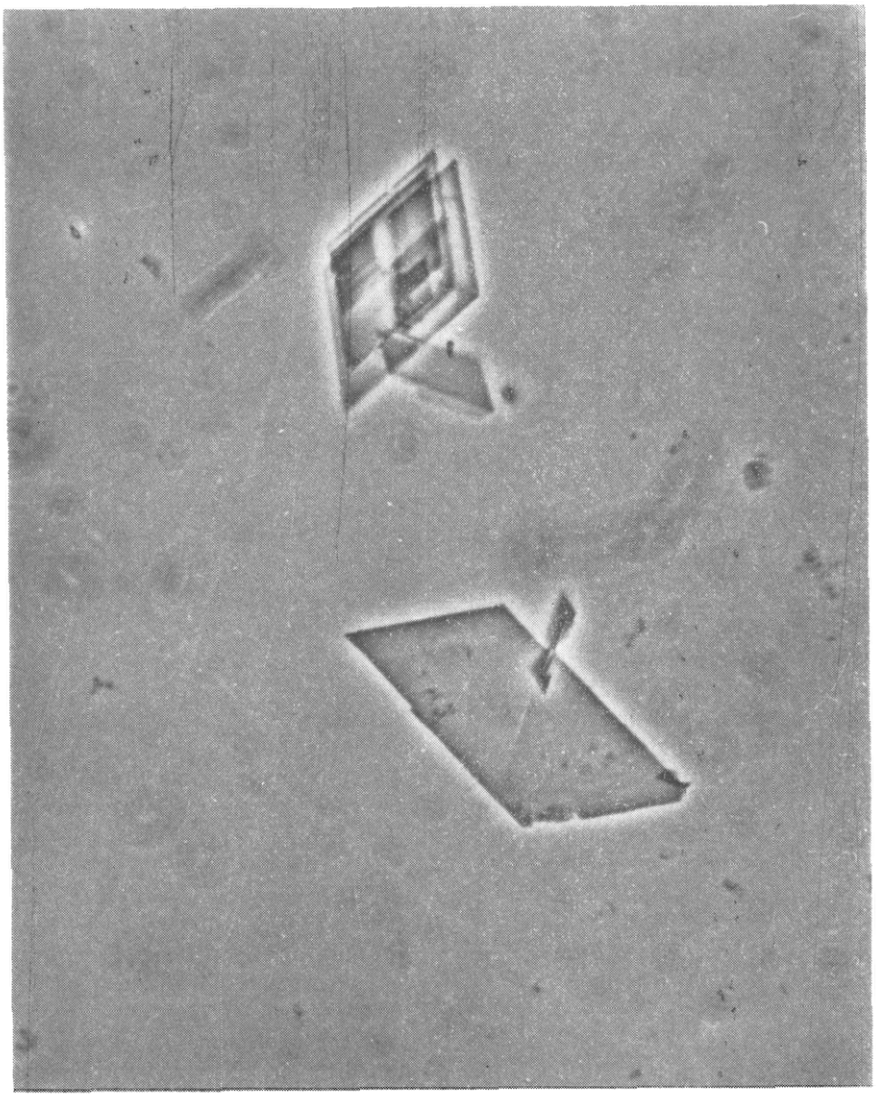

FIGURE 1: Crystalline aspartate holoaminotransferase. Crystallized from 0.05 M potassium phosphate buffer (pH 7.5); magnification ×400.

Martinez-Carrion and Tiemeier, 1967). A similar situation is also present in the soluble and mitochondrial avian enzymes.

Figure 2 shows the starch gel pattern of chicken heart aspartate aminotransferase. The soluble enzyme migrates toward the anode; the mitochondrial enzyme migrates to the cathode. The soluble AAT is composed of more than six enzymatically active proteins in the crude extract. The distribution of these bands of activity is not altered by purification and is the same in both the holo- and apoenzymes. Protein stains of the starch gels showed that there were no protein components in the crystalline soluble AAT which were not enzymatically active. The proteins which compose the soluble chicken heart AAT were separated by column chromatography (procedure to be presented elsewhere). On starch gel electrophoresis, the separated proteins move in the same position as they did in the original mixture.

The mitochondrial AAT is also composed of several bands of activity in crude extracts. Purification and crystallization of this enzyme (procedure to be presented elsewhere) did not alter the appearance of the electrophoretic pattern.

Extinction Coefficient. The extinction coefficients of the holo- and apo-AAT were determined in the Model E ultracentrifuge using the double-sector synthetic-boundary cell and the interference optics (Richards and Schachman, 1959). Bovine serum albumin ($\epsilon_{278}^{1\%}$ 6.67) was used as the standard (Foster and Sherman, 1956). A solution of bovine serum albumin of known optical density was used to determine the concentration of protein in milligrams per milliliter which one interference fringe represented. This value was then used to determine the concentration of protein present in an AAT solution of known optical density. The $\epsilon_{280}^{1\%}$ of holo-AAT in 0.05 M potassium phosphate buffer (pH 7.5) is 15; that of apo-AAT is 14.5 in the same buffer.

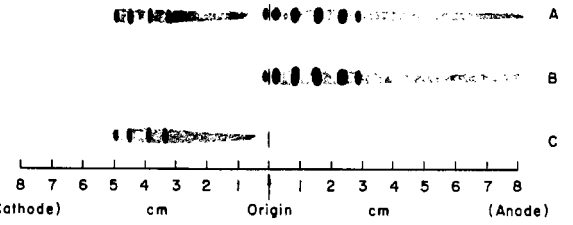

FIGURE 2: Starch gel electrophoretic pattern of chicken heart aspartate aminotransferase. The gel was stained for enzymatic activity. (A) Crude chicken heart extract, (B) crystalline soluble AAT, and (C) crystalline mitochondrial GOT.

Resolution of Holoaminotransferase. Pyridoxal phosphate and pyridoxamine phosphate were found to restore full catalytic activity to the apoenzyme. Both forms of the coenzyme were tightly bound and could not be removed by prolonged dialysis. The holoenzyme could be resolved by a procedure similar to that of Scardi *et al.* (1963). The enzyme was first incubated with a one thousand fold excess of aspartate for 15 min. The enzyme solution was brought to 0.5 M in phosphate by the addition of 1 M potassium phosphate buffer (pH 5.5) and, if necessary, the pH of the resulting solution was adjusted to 5.5. This mixture was incubated at 30° for 55 min and then dialyzed against 0.1 M potassium phosphate buffer (pH 6.5). This treatment resulted in 92–100% resolution with a recovery of 80–90% of the initial activity.

Stability of Holo- and Apoaminotransferases. The apoaminotransferase was markedly less stable than the holoenzyme to a variety of denaturing agents. The apoenzyme, in 8 M urea, lost one-half of its activity in 2.5 min while the holoenzyme lost one-half of its activity in 51.5 min. The holoenzyme was less resistant to denaturation by extremes of acid and alkaline pH than the apoenzyme (Figure 3).

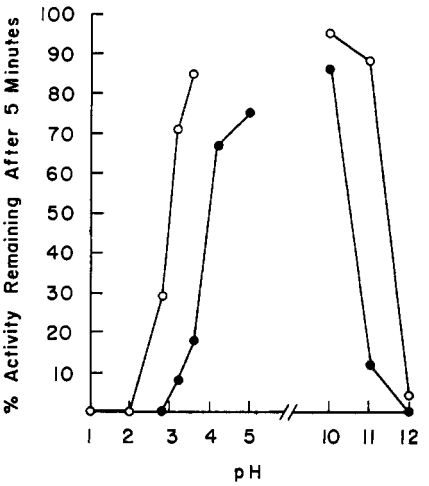

FIGURE 3: pH stability of chicken heart soluble AAT. (O—O) apo-AAT; (O—O) holo-AAT. The enzyme concentration in the incubation mixture was 2×10^{-3} mg/ml; temperature, 22°. Buffers 0.05 M KCl–HCl, (pH 1.0 and 2.0), 0.05 M glycine–HCl (pH 2.8, 3.2, 3.6, 4.2, and 5.0), and 0.05 M glycine–KOH (pH 10.0, 11.0, and 12.0).

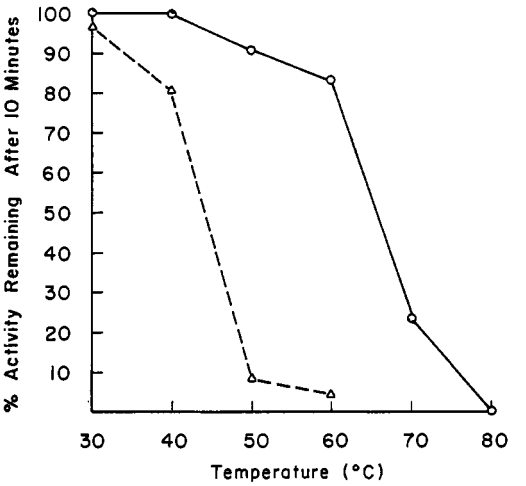

FIGURE 4: Heat denaturation of chicken heart soluble AAT. The incubation mixture contained 2×10^{-3} mg of enzyme/ml of 0.1 M Tris-HCl (pH 7.4). (Δ - - Δ) apo-AAT and (O—O) holo-AAT.

The apoenzyme was markedly less stable than the holoenzyme to denaturation by heat (Figure 4). Both the holo- and apoenzymes were protected by α-ketoglutarate, maleate, glutarate, and citrate (Table II). Aspartate had no effect on the apoenzyme and destabilized the holoenzyme. The apoenzyme was strongly protected by phosphate and pyrophosphate which had no effect on the holoenzyme. Arsenate, sulfate, and nitrate protected the apoenzyme to a lesser extent than did phosphate.

Absorption Spectra. The holoenzyme in 0.05 M sodium citrate buffer (pH 5.0) has an absorption maximum at 430 mμ; in 0.05 M potassium phosphate buffer (pH 7.5) the absorption maximum shifts to 360 mμ. Addition of a 50-fold excess of L-aspartate to the enzyme shifts the absorption maximum to 333 mμ at both of these pH values. The values are quite similar to those which have been observed with the pig heart soluble AAT (Jenkins *et al.*, 1959).

Fluorescence Spectra. Solutions of holo- and apo-AAT in 0.1 M Tris-HCl buffer (pH 7.4) with an optical density of 0.12 at 280 mμ were excited at 280 mμ. Both proteins had emission maxima at 335 mμ; however, the tryptophan fluorescence intensity of the holoenzyme has markedly decreased (Figure 5). No energy transfer was observed between the tryptophan and the bound coenzyme.

The holo- and apoenzymes also differ in their polarization of tryptophan fluorescence. Enzyme solutions of 0.05 mg/ml in 0.1 M Tris-HCl buffer (pH 7.4) were excited at 280 mμ, and their polarization of fluorescence

TABLE II: Heat Stability of Chicken Heart Soluble Aminotransferase.[a]

Addition	Apo-S-AAT $t_{1/2}$ (°C)	Holo-S-AAT $t_{1/2}$ (°C)
None	42.7 ± 2	66.0 ± 1
2.5×10^{-3} α-keto-glutarate	51.2	72.7
2.5×10^{-3} glutarate	48.7	72.9
2.5×10^{-3} maleate	54.5	72.7
2.5×10^{-3} citrate	58.5	69.3
2.5×10^{-3} aspartate	45.2	63.9
2.5×10^{-3} potassium phosphate	62.6	66.2
2.5×10^{-4} potassium phosphate	56.0	
2.5×10^{-4} sodium pyrophosphate	59.5	
2.5×10^{-5} potassium phosphate	54.5	
2.5×10^{-3} potassium arsenate	59.6	
2.5×10^{-3} potassium sulfate	53.6	
2.5×10^{-3} potassium nitrate	47.5	
2.5×10^{-3} potassium acetate	42.0	
2.5×10^{-3} potassium chlorate	42.0	
1.0×10^{-3} pyruvate	42.0	

[a] The final concentration of enzyme in the incubation mixture was 3 μg/ml. All compounds were adjusted to pH 7.4 before use. $t_{1/2}$ is defined as the temperature at which one-half of the initial activity is lost after a 10-min incubation.

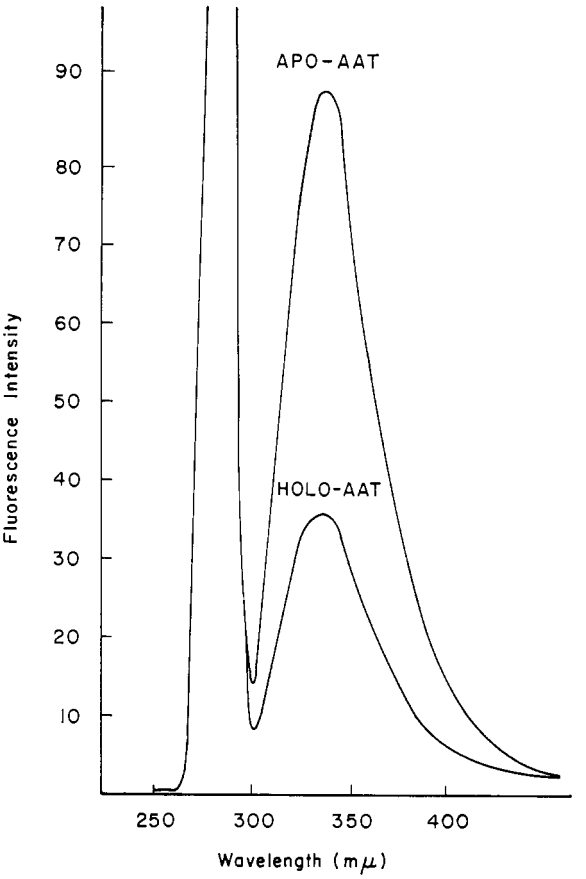

FIGURE 5: Fluorescence emission spectra of holo- and apo-AAT. Excitation at 280 mμ. The optical density of the protein at 280 mμ is 0.12 in 0.1 M Tris-HCl buffer (pH 7.4).

was measured at 335 mμ. The apoenzyme has a value for the polarization (p) of 0.22; the holoenzyme has a p of 0.37.

Titration of Apoaminotransferase with Coenzyme. The decrease in holoenzyme fluorescence intensity at 335 mμ and the increase in polarization of fluorescence provide a sensitive method for the determination of the stoichiometry of the coenzyme–enzyme binding. Apoenzyme solutions containing 0.05–0.1 mg/ml of apoenzyme in 0.1 M Tris-HCl (pH 7.4) were used for the fluorescence titrations. Aliquots (2 μl) of coenzyme solution (2.5×10^{-4} M) were added to the enzyme solution, and the fluorescence and polarization of fluorescence were measured at 335 mμ after each addition. Due to the slow rate of binding of the coenzyme, it is necessary to incubate the enzyme and coenzyme for 15 min before maximum binding occurs.

Figure 6 illustrates the titration of the apoenzyme with pyridoxal phosphate. Corrections were made for volume changes. The equivalence point for fluorescence titrations was determined from a plot of $1/\Delta F$ *vs.* 1/[coenzyme]; for polarization of fluorescence titrations, the equivalence point was determined from a plot of p *vs.* [coenzyme]. Four fluorescence titrations with pyridoxal phosphate gave a bound value of 52,800 ± 500 g of enzyme/mole of coenzyme; two fluorescence titrations with pyridoxamine phosphate gave a value of 48,900 ± 4000 g/mole of coenzyme. Two polarizations of fluorescence titrations with pyridoxal phosphate gave values of 48,700 ± 4000 g/mole of coenzyme.

The amount of pyridoxal phosphate bound was also determined chemically by the phenylhydrazine method of Wada and Snell (1963). Two determinations each gave a value of 46,900 g of enzyme protein/mole of coenzyme.

Molecular Weight of Aspartate Aminotransferase. The dependence of the sedimentation coefficient on protein concentration was determined at protein concentrations from 0.09 to 0.72% in 0.05 M potassium phosphate buffer (pH 7.5) at 22°. There was a slight negative slope in the plot of $s_{20,w}$ *vs.* concentration, and the value of 5.5

TABLE III: Sedimentation Equilibrium Ultracentrifugation of AAT in Dilute Solution.

Buffer (M), pH	Protein Concn (mg/ml)	Speed (rpm)	Mol Wt
Potassium phosphate (0.05), 7.5	0.800	17,980	100,000
	0.069	17,980	92,400
	0.030	17,980	87,950
Glycine–KOH (0.05), 9.0	0.750	29,500	90,500
	0.060	29,500	73,300
Glycine–KOH (0.05), 10.0	0.750	17,980	90,500
	0.075	29,500	71,700

S was determined by extrapolation to zero concentration. The sedimentation coefficients of the apoenzyme fall within the same range as those of the holoenzyme. The sedimentation equilibrium molecular weight of the holoenzyme was found to be 100,000 at a concentration of 0.8 mg/ml in 0.05 M potassium phosphate buffer (pH 7.5) (Table III).

Dissociation of Aspartate Holoaminotransferase into Subunits. A. ACID DISSOCIATION. Attempts to dialyze the enzyme into low pH buffers resulted in the formation of aggregates with an $s_{20,w}$ of 15. Partial dissociation of the enzyme was obtained by diluting a 1.7% solution of protein in 0.05 M potassium phosphate (pH 7.5) to a final concentration of 0.22% with 0.05 M glycine–HCl buffer (pH 3.0). A sedimentation velocity run at 22° 2 hr after dilution showed the presence of approximately 30% of a component with an $s_{20,w}$ of 2.56 and 70% of a 5.42S component. After 48 hr, the enzyme had been completely converted to the 2.56S form. Complete dissociation was brought about by titrating an enzyme solution

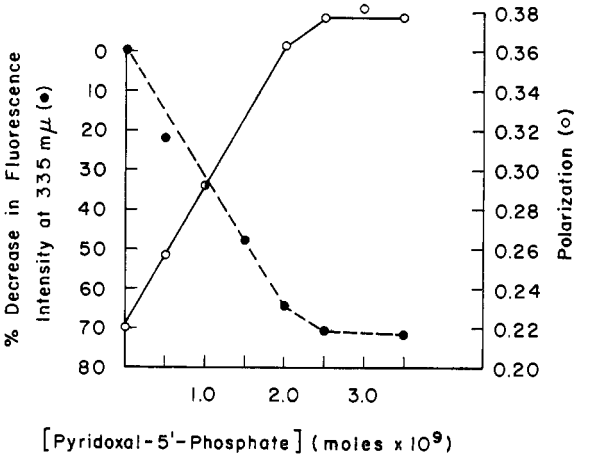

FIGURE 6: Titration of apo-AAT with pyridoxal phosphate. (○—○) Polarization of fluorescence and (●—●) fluorescence. The enzyme concentration is 0.096 mg/ml of 0.1 M Tris-HCl buffer (pH 7.4). Excitation is at 280 mμ; temperature, 24°.

in 0.05 M potassium phosphate buffer (pH 7.5–3.0) with 0.1 N HCl. At this pH, a sedimentation equilibrium run at 29,500 rpm gave a weight-average molecular weight of 57,500 for the protein.

B. DISSOCIATION IN DILUTE SOLUTION. The sedimentation properties of very dilute solutions of the enzyme were examined by the use of the highly sensitive interference optics of the ultracentrifuge. Sedimentation equilibrium ultracentrifugation of the holoaminotransferase was carried out at protein concentrations from 0.003 to 0.07% at pH 7.5, 9.0, and 10.0 (Table III). At pH 7.5, a small dissociation of the 100,000 mol wt enzyme occurs, but an increase in pH brings about a much larger degree of dissociation of the enzyme into monomer.

Aspartate Aminotransferase Tetramer. Chromatography of the purified holoenzyme preparation on Sephadex G-100 showed the presence of a protein component which had the same specific activity as the 5.5S enzyme, but which had a higher molecular weight. The amount of this form of the enzyme obtained varied from preparation to preparation. This component had an $s_{20,w}$ of 8.1 and a sedimentation equilibrium molecular weight of 189,000 at pH 7.5 indicating that it was a tetramer of the 2.45S subunit. The 8.1S enzyme had a pattern on starch gel identical with that of the 5.5S enzyme.

The 8.1S enzyme could be dissociated in 0.01 M 2-mercaptoethanol to the 5.5S enzyme; even after prolonged incubation in mercaptoethanol no dissociation to the 2.5S form was observed. When the mercaptoethanol was removed by dialysis, 10% of the enzyme reverted back to the 8.1S form. Attempts to produce the 8.1S enzyme from the native 5.5S form by treatment with $(NH_4)_2SO_4$ or by incubation at pH 10 were unsuccessful.

An attempt was made to dissociate the tetramer using the same conditions of low concentration and high pH which dissociated the 5.5S enzyme. Sedimentation equilibrium ultracentrifugation of a 0.02% solution of the tetramer in 0.05 M glycine–KOH buffer (pH 10.0) gave a value of 250,000 for the weight-average molecular weight indicating that no dissociation had occurred. The increase in molecular weight could possibly arise from further aggregation of the tetramer under these conditions.

Discussion

The results presented here indicate that the binding of either form of the coenzyme to aspartate aminotransferase is accompanied by a change in the conformation of the apoenzyme molecule. The increased stability of the holoenzyme to acid, base, and urea indicates that the holoenzyme structure is less accessible to attack by these agents.[2]

Pyridoxal phosphate confers a great degree of thermal stability upon the apoenzyme. This effect is mimicked by pyrophosphate, phosphate, and related anions. It is interesting that phosphate has no protective effect upon the holoenzyme implying that the phosphate moiety of the coenzyme plays a major role in the protective effect of the coenzyme. Ketoglutarate and related inhibitors of the enzyme stabilize both the holo- and apoenzymes. Aspartate, however, destabilizes the holoenzyme; this is probably the result of a partial removal of the coenzyme by aspartate.

The increased polarization of fluorescence of the holoenzyme indicates that the binding of the coenzyme changes the structure of the apoenzyme in such a way as to make the protein a more rigid and ordered structure. Optical rotatory dispersion studies of pig heart AAT also demonstrate that the binding of the coenzyme produces a conformational change in the apoenzyme molecule (Fasella and Hammes, 1964, 1965).

The stoichiometry of coenzyme binding has been determined by measurement of the decrease in tryptophan fluorescence which occurs upon binding of either form of the coenzyme to the apoenzyme. This method avoids errors which arise from photodecomposition of the free coenzyme when coenzyme fluorescence is measured by excitation at 330 mμ (Bridges *et al.*, 1966). Quenching of the tryptophan fluorescence occurs upon coenzyme binding, but this is not accompanied by energy transfer between the tryptophan and the coenzyme. According to the theory of Förster (1959), energy transfer between two species is a function of the lifetime of the excited state, the orientation of the two species, and the degree of overlap of their absorption and fluorescence spectra. Since the absorption spectra of both enzyme-bound pyridoxamine phosphate and pyridoxal phosphate overlap the fluorescence emission spectrum of tryptophan, the situation would be very favorable to energy transfer. The absence of energy transfer implies that the orientation of the bound coenzyme is unfavorable for this type of interaction with the tryptophan residues. This situation has been thoroughly discussed by Churchich (1965) who has observed energy transfer in conjugates of pyridoxamine phosphate and human serum albumin, lysozyme, and carboxypeptidase but not with pig heart aspartate aminotransferase.

The molecular weight of the chicken heart soluble aminotransferase based on sedimentation equilibrium ultracentrifugation and on the stoichiometry of coenzyme binding is 100,000 ± 5000. This is slightly lower than the molecular weight of 110,000 reported for the pig heart enzyme (Jenkins *et al.*, 1959) and of the same order as the molecular weights of 95,000 of rat liver AAT (Harpring, 1965) and 96,000 for ox heart AAT (Marino *et al.*, 1966). The presence of very small amounts of the tetramer or dissociation of the enzyme into subunits in dilute solution may be a complicating factor in the determination of the exact molecular weight of the enzyme by ultracentrifugal methods.

The dissociation of the chicken heart AAT into subunits at acid pH is similar to that reported by Polyanovsky and Shpikiter (1965) for pig heart AAT. Polyanovsky (1965) has also reported that succinylated AAT in dilute solution dissociates at alkaline pH. Sedimentation equilibrium ultracentrifugation of the chicken heart AAT has shown that the 100,000 mol wt form of the enzyme does dissociate at pH 10; however, since the enzymatically active tetramer shows no dissociation under the same conditions, it would seem that dissociation into the 50,000 mol wt subunit is not a prerequisite for enzymatic activity.

At the present time, the factors causing the formation of the tetramer are not known. A similar minor, high molecular weight rat liver alanine aminotransferase has been observed (L. Bertland and M. Wanta, unpublished observations). The chicken tetramer is apparently formed under those conditions which would be favorable to the formation of intramolecular disulfide bonds between the native enzyme molecules. Possibly there may be a subtle difference in the amino acid sequence of those molecules of enzyme which form the tetramer which allows them to form the higher molecular weight structure. No evidence has been found that would indicate disulfide bonds to exist between the two subunits of the 100,000 mol wt form of the enzyme.

[2] The protection by the coenzyme against heat and denaturing reagents may also be due to a shielding of a critical area of the transaminase.

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