Studies on the Conformations of the Multiple Forms of Chicken Heart Aspartate Aminotransferase*

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ABSTRACT: Crystalline chicken heart soluble aspartate aminotransferase (hereafter called the "soluble" enzyme) and mitochondrial aspartate aminotransferase (hereafter called the "mitochondrial" enzyme) have different amino acid compositions, kinetic properties, and heat stabilities. The circular dichroism of the mitochondrial enzyme shows negative bands at 208 and 222 mμ suggesting an α-helical structure. The soluble enzyme has a negative band at 217 mμ indicating a β structure; the soluble enzyme infrared spectrum has a maximum at 1652 cm^{-1} and also distinct peaks at 1637 and 1685 cm^{-1} indicative of a β structure. The soluble enzyme is composed of three proteins, designated α, β, and γ in order of increasing negative charge. These proteins are present in extracts of fresh hearts; however their distribution and number is altered by the purification procedure. These proteins have identical molecular weights, amino acid compositions, kinetic properties, and heat stabilities. The increase in the negative charge of the protein correlates with a decrease in the intensity of the ultraviolet circular dichroism, a decrease in the fluorescence of the tryptophan residues, and an increase in the 250-mμ and 300–340-mμ absorption. In 6 M guanidine hydrochloride, the soluble enzyme completely loses activity. The activity may be recovered only in the presence of pyridoxal phosphate and mercaptoethanol. Reactivation does not alter the electrophoretic pattern of the soluble aspartate aminotransferase proteins.

The presence in a single cell of several proteins with the same catalytic activity provides an excellent system for the study of the relationship of primary structure, tertiary conformation, and kinetic properties. Aspartate aminotransferase [L-aspartate:2-oxoglutarate aminotransferase (EC 2.6.1.1)] is a system in which there are two principle proteins from different cellular locations and apparently under different genetic controls. Each of these two main protein fractions is composed of two or more electrophoretically different proteins.

The two main forms of aspartate aminotransferase in rat liver were found to originate from the soluble and mitochondrial cell fractions (Boyd, 1961). These two forms occur in a number of tissues and have been found to be present in dog heart (Fleisher *et al.*, 1960), beef heart, pig heart (Wada and Morino, 1964), chicken heart (Bertland and Kaplan, 1968), and human tissues (Bodansky *et al.*, 1966). The pig heart mitochondrial and soluble enzymes have different kinetic properties and immunological reactions (Wada *et al.*, 1968), as well as a different primary structure (Martinez-Carrion and Tiemeier, 1967). Studies on the concentration of these two enzymes at various stages of growth, (Nakata *et al.*, 1964; Scheid *et al.*, 1965) and on induction of these enzyme, (Katunuma *et al.*, 1966, 1968) suggest that the soluble and mitochondrial aspartate aminotransferases (hereafter referred to as soluble and mitochondrial enzymes) are under separate genetic control. This conclusion is substantiated by different N-terminal amino acids in the soluble and mitochondrial pig heart enzymes (Martinez-Carrion and Tiemeier, 1967; Wada *et al.*, 1968). A recent study by Morino and Watanabe (1969) has indicated that the amino acid sequence of the soluble and mitochondrial pig heart aminotransferases differ in one or more amino acids although both contain an asparagine residue in proximity to the lysine and which binds the pyridoxal phosphate.

The microheterogeneity of (anionic) rat liver soluble enzyme was first demonstrated by Decker and Rau (1963). The multiple forms of the pig heart soluble enzyme have been studies extensively by Martinez-Carrion *et al.* (1965, 1967). The data of these authors stongly suggests that the primary structure of the three major protein components of pig heart soluble enzyme are quite similar in regard to amino acid composition, amide nitrogen, and amino acid sequence. These authors found that the significant differences between the proteins were their electrophoretic mobility, stability in 8 M urea, and particularly, in their rate and mode of pyridoxal phosphate binding. It was ascertained that the most anionic pig heart soluble protein has its coenzyme bound in a manner which renders it incapable of reacting with amino acid substrates. This pyridoxal phosphate, which has been designated as catalytically inactive, is characterized by an absorption maximum at 340 mμ. Catalytically active pyridoxal phosphate (that coenzyme which is fully capable of carrying out the transamination reaction) is characterized by absorption maxima at 360 mμ with a high pH and 430 mμ at a low pH. The catalytically inactive pyridoxal phosphate does not account for the difference in electrophoretic mobility since the less anionic proteins may also contain this material without alteration of their electrophoretic properties.

* Publication No. 716 from the Graduate Department of Biochemistry, Waltham, Massachusetts 02154. *Received October 3, 1969.* This investigation was supported by Public Health Service Research Grant No. CA-03611 from the National Cancer Institute and American Cancer Society Grant No. P-77K.

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A similar study has been conducted on the three subforms of pig heart mitochondrial aspartate aminotransferase by Michuda and Martinez-Carrion (1969) with quite similar results. They found that the mitochondrial proteins do not differ in amino acid composition, amino acid sequence, molecular weight, and optical rotatory dispersion in the ultraviolet region. Again, the only difference was the amount of catalytically inactive pyridoxal phosphate bound.

Bossa *et al.* (1968) reported the presence of a second set of pig heart soluble proteins which are similar to the proteins described above, but which have a high carbohydrate content.

We have found that chicken heart aspartate aminotransferase is also composed of two principle fractions: anionic (soluble) and cationic (mitochondrial). Both of these proteins display heterogeneity on starch gel electrophoresis. The gel pattern of the crystalline mitochondrial enzyme is identical with that in the crude extract, but the gel pattern of the partially purified and crystalline soluble enzyme differs from that of the crude extract in both the distribution and number of bands. We found that there are three bands of soluble enzyme activity present in crude extracts of the supernatant fractions of chicken heart and we have designated these α, β, and γ, in order of their increasing anionic mobility; these correspond to the nomenclature applied to subforms of the pig heart enzyme (Martinez-Carrion *et al.*, 1967).

This paper presents an examination of the physical and kinetic properties of the multiple forms of chicken heart aspartate aminotransferase. We present here a study of the amino acid composition, visible and ultraviolet spectra, circular dichroism, and related kinetic properties of the mitochondrial and soluble enzymes, as well as the various subforms which compose the soluble aminotransferase.

Materials

Chicken hearts were obtained from a slaughterhouse or a local market. L-Aspartic acid and pyridoxal phosphate were from Mann Research Co. Malic dehydrogenase and α-ketoglutaric acid were from California Corporation for Biochemical Research. Pyridoxine and pyridoxal were obtained from Sigma. Azoene fast violet B salt was purchased from Alliance Chemical Corp. Sephadex G-100 was obtained from Pharmacia Fine Chemicals. Whatman DE-11, DE-52, and CM-11 were products of Reeve Angel Company. Hydrolyzed starch for gel electrophoresis was obtained from Connaught. Guanidine was purchased from Eastman, and was recrystallized before use. 2-Mercaptoethanol was obtained from Eastman. All other chemicals used were of reagent grade.

Methods

Determination of Enzymatic Activity. Aspartate aminotransferase activity was measured as described previously (Bertland and Kaplan, 1968). Protein concentrations of the soluble, α-soluble, β-soluble, γ-soluble, and mitochondrial mixtures were routinely determined by measuring the absorption at 280 mμ in either 0.1 M Tris-HCl buffer, pH 7.4, or 0.05 M potassium phosphate, pH 7.5. The value of 14 for the extinction coefficient of a 1% solution of aminotransferase in a 1-cm path length was used for the calculation of protein concentration of all the aspartate aminotransferase proteins. This value was determined by measuring the protein concentration with the Rayleigh optics of the analytical ultracentrifuge as a refractometer (Bertland and Kaplan, 1968). The extinction coefficients of the individual proteins were also measured by determining the ratio of their absorption at 280 mμ to the protein concentration determined by the colorimetric method of Sutherland *et al.* (1949). This method led to the following values for $\epsilon^{1\%}_{280\,m\mu}$: soluble mixture, 14.2; α soluble, 13.7; β soluble, 14.5; γ soluble, 14.0; mitochondrial, 13.2.

Amino Acid Analysis. Amino acid analyses were performed in a Beckman–Spinco automatic amino acid analyzer. Samples were dialyzed extensively against distilled water, and were hydrolyzed in constant boiling point HCl in evacuated sealed vials for 24, 48, and 72 hr at 110° (Moore *et al.*, 1958). Tryptophan was determined by the method of Spies and Chambers (1949).

Starch Gel Electrophoresis and Ultracentrifugation. These techniques were carried out by methods described previously (Bertland and Kaplan, 1968).

Spectra. Circular dichroism studies were done on a Cary Model 60 recording spectropolarimeter with circular dichroism accessory Cary Model 6001. Fluorescence was measured with a Turner Model 210 "spectro" absolute spectrofluorometer (Turner, 1964). Absorption spectra were measured on a Cary 14 spectrophotometer or a Zeiss PMQ II spectrophotometer. The sample for infrared analysis was prepared by depositing enzyme solution on a silver chloride pellet, and removing the water *in vacuo*. The infrared spectra were measured on a Perkin-Elmer 221 spectrophotometer.

Preparation of Enzymes. Chicken heart soluble enzyme was prepared as described previously (Bertland and Kaplan, 1968). At first, enzyme solutions were concentrated by placing the solution in a dialysis bag and placing the bag in reagent grade sucrose. The sucrose which entered the enzyme solution was then removed by dialysis. Later the enzyme solutions were concentrated with lypogel (Gelman Instrument Co.).

The apoenzymes were initially prepared by the method of Scardi *et al.* (1963). Later, it was more convenient to remove the coenzyme by dialysis of the enzyme solution against 1000 volumes of 0.05 M potassium phosphate buffer–0.01 M L-aspartate (pH 5.5) for 20 hr, followed by dialysis against any desired buffer. This method yields 99.9 to 100% resolution with 90% recovery of activity.

Mitochondrial enzyme was isolated as a by-product from a procedure designed for the simultaneous purification of chicken heart lactic dehydrogenase, malic dehydrogenase, and soluble aspartate aminotransferase. Some mitochondrial enzyme activity is present in the first 0–70% ammonium sulfate precipitate; this activity may be recovered from the CM-11 column in step five of the soluble enzyme purification procedure of Bertland and Kaplan (1968).

All steps in the procedure were carried out at 4°; all dialyses were carried out against three changes of 10–100 volumes of buffer for a period of 12–16 hr. A summary of the purification procedure for the mitochondrial enzyme is given in Table I.

Step 1. Frozen chicken hearts were minced and extracted for 1 hr with cold distilled water (0.56 l./lb). The extract was filtered through cheesecloth and centrifuged for 30 min at 1300*g*.

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

Step	Total Activity (units)	Total Protein (mg)	Sp Act. (units/ OD_{280})	Total Yield (%)
1. Crude extract	31.6×10^6	9.2×10^5	34	
2. 70–90% Ammonium sulfate	4.32×10^6	1.5×10^4	284	13.7
3. 30–50% Ammonium sulfate	1.54×10^6	2.3×10^3	675	4.8
4. Chromatography on CM-11	1.11×10^6	435	2560	3.5
5. 0–80% Ammonium sulfate	33.3×10^4	179	1865	1.1
6. Chromatography on Sephadex G-100	23.4×10^4	78	3000	0.7
7. First crystallization	8.75×10^4	19.2	4820	0.3

[a] Summary of the purification steps in the preparation of mitochondrial aspartate aminotransferase from 22 kg of chicken hearts as described in the text.

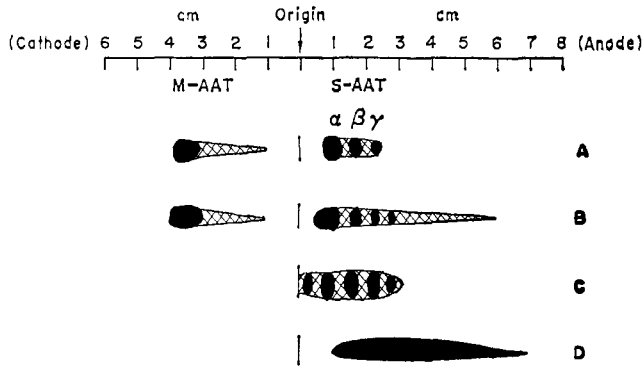

FIGURE 1: Starch gel electrophoretic pattern of chicken heart aspartate aminotransferase at different stages of the purification procedure. The gels were stained for enzymatic activity: (A) fresh chicken heart extract; (B) first 0–80% ammonium sulfate precipitate after 12 days; (C) soluble enzyme eluted from DE-11 column with 0.005 M potassium phosphate buffer (pH 7.5); (D) soluble enzyme eluted from DE-11 column with 0.005 M potassium phosphate buffer (pH 7.5)–0.2 M NaCl.

Step 2. Solid ammonium sulfate was added to the supernatant to a concentration of 70%, and the precipitate was removed by filtration. The 70% supernatant was brought to a 90% ammonium sulfate concentration, allowed to stand for 24 hr, and filtered through fluted filter paper.

Step 3. The 90% precipitate was redissolved in enough 0.05 M potassium phosphate buffer (pH 6.5) to give a clear solution. The following ammonium sulfate fractions were then taken: 0–30, 30–40, 50–60, and 60–90%. After adding the ammonium sulfate, the solution was always left standing for 30 min and then centrifuged. The precipitates were dissolved in a minimal volume of 0.05 M potassium phosphate buffer (pH 6.5). The fraction(s) having the highest specific activities were pooled and dialyzed against 0.005 M potassium phosphate buffer (pH 6.5)–0.001 M EDTA.

Step 4. A carboxymethylcellulose column was prepared containing 1 ml of packed resin/5 mg of protein in the dialyzed enzyme solution. The resin was equilibrated with 0.005 M potassium phosphate buffer (pH 6.5)–0.001 M EDTA. After application of the dialyzed enzyme solution, the column was eluted with the starting buffer in order to remove nay soluble enzyme present in the enzyme solution. When protein was no longer removed from the column, a linear gradient was applied consisting of two column volumes of 0.005 M potassium phosphate buffer (pH 6.5)–0.001 M EDTA in the mixing flask, and two column volumes of 0.1 M potassium phosphate buffer (pH 6.5)–0.001 M EDTA in the reservoir flask. The mitochondrial enzyme fractions with the highest specific activity were pooled. Ammonium sulfate was added to a final concentration of 80%, and the solution was allowed to stand overnight. The precipitate was collected by centrifugation, and dissolved in a minimum volume of 0.05 Tris-HCl buffer (pH 7.5) and then dialyzed against this buffer.

Step 5. The dialyzed enzyme was applied to a Sephadex G-100 column equilibrated with 0.05 M Tris-HCl buffer (pH 7.5)–0.1 M KCl. The height to diameter ratio of the column was 15:1, and the volume was 10 to 20 times that of the enzyme solution. The enzyme was eluted from the column with 0.05 M Tris-HCl buffer (pH 7.5)–0.1 M KCl. The fractions with the highest specific activity were pooled.

Step 6. If the protein concentration at this point was not at least 2 mg/ml or higher, the enzyme solution was concentrated and reequilibrated with 0.05 M Tris-HCl buffer (pH 7.5)–0.1 M KCl. Finely powdered ammonium sulfate was then added to a final concentration of 50% and the solution was centrifuged. Ammonium sulfate was then added slowly, until faint turbidity appeared; this was removed by centrifugation, and the solution was then allowed to stand for 16 hr at 4°. If crystallization did not occur, ammonium sulfate was again added until turbidity formed, the precipitate was removed, and the solution was allowed to stand. This was repeated until needle-shaped crystals were formed. Recrystallization was carried out in the same manner. The maximum specific activity of the crystalline enzyme was 4820 units/ $OD_{280\,m\mu}$. This enzyme was homogeneous as it gave a single sharp peak in the ultracentrifuge at a protein concentration of 6.1 mg/ml. If the mitochondrial enzyme is stored as an 80% ammonium sulfate precipitate, it is stable for a year.

Results

Properties of the Multiple Forms of Aspartate Aminotransferase in Crude Extracts. The following experiments were conducted in order to determine: (a) whether the multiple forms of the enzyme were present in the intact cell; (b) if freezing the heart had any effect on the various enzyme forms; and (c) if the purification procedure altered the electrophoretic distribution of the multiple forms.

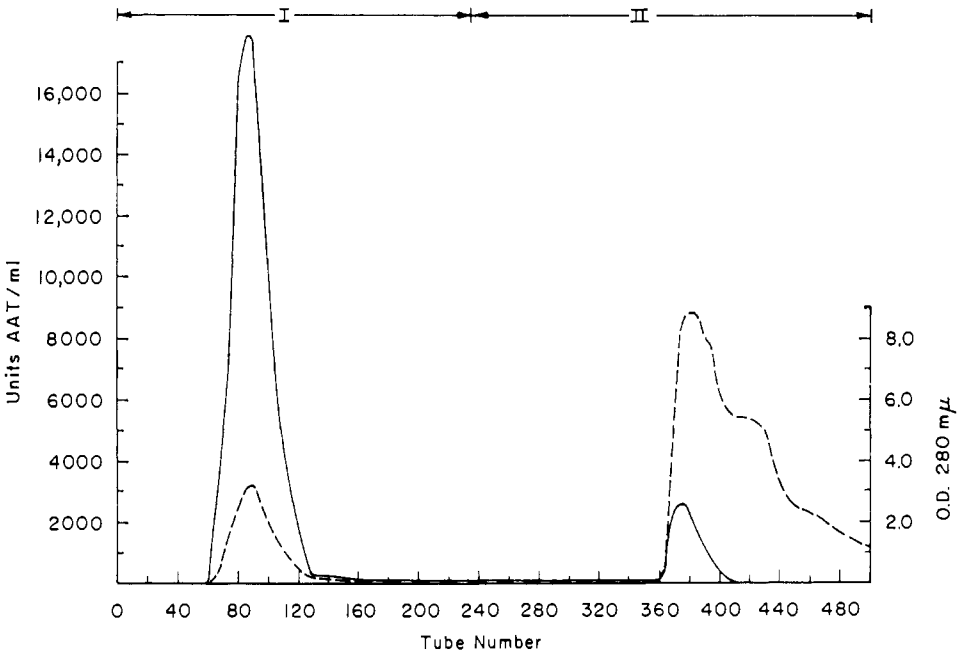

FIGURE 2: Chromatography of chicken heart soluble aspartate aminotransferase on DE-11. Soluble enzyme (11.6×10^5 units) was applied to the column which contained 2.5 l. of packed DE-11 equilibrated with 0.005 M potassium phosphate buffer (pH 7.5). Fractions (14-ml) were collected. The solid line represents units per milliliter. The broken line represents optical density at 280 mμ: (I) elution with 0.005 M potassium phosphate buffer (pH 7.5); (II) elution with 0.005 M potassium phosphate buffer (pH 7.5)–0.2 M NaCl.

Four chickens were killed, and their hearts were removed immediately. A 1-g portion of each heart was minced, homogenized individually in a hand homogenizer with 5 ml of cold distilled water, and centrifuged. The level of the enzyme was 2000 units/g of heart, and the enzymes contained 90–94% of the pyridoxal phosphate required for maximum catalytic activity. The amount of pyridoxal phosphate bound to the enzyme was determined for activity measurements in the presence and absence of pyridoxal phosphate. Part of each extract was concentrated with sucrose. Gel electrophoresis of both the concentrated and unconcentrated extracts showed identical patterns (Figure 1). The soluble enzyme had three bands of activity, and the mitochondrial preparation gave a smeared but heterogeneous pattern (Figure 1A). All four hearts analyzed show identical electrophoretic patterns.

Frozen hearts (obtained from a local market) were extracted in the same manner as described above. The activity per gram of heart was approximately the same as that in the fresh heart, and the starch gel pattern observed was the same. The amount of pyridoxal phosphate bound, however, was lower. Due to the fact that the coenzyme is very tightly bound to the enzyme (Bertland and Kaplan, 1968), it was of interest to determine if there was a selective loss of coenzyme from one of the multiple forms. The extract (1 ml) was dialyzed against 0.005 M potassium phosphate buffer (pH 6.5)–0.001 M EDTA and chromatographed on a CM-11 column (1 × 12 cm) which was equilibrated with the same buffer. The soluble enzyme was not retained by the column; the mitochondrial enzyme was bound to the column, and was eluted with 0.1 M potassium phosphate buffer (pH 6.5)–0.001 M EDTA. Fractions (2 ml) were collected. Starch gel electrophoresis indicated that the separation of the two types of enzymes was complete. The column fractions were assayed both with and without pyridoxal phosphate. Only the soluble enzyme had lost pyridoxal phosphate upon freezing (approximately 50%), whereas the mitochondrial enzyme still contained all its coenzyme bound.

The extract from the frozen heart was placed on a starch gel. The gel was sliced in half horizontally; one half was developed with pyridoxal phosphate in the staining solution, while the other half was developed without the pyridoxal phosphate. The soluble enzyme pattern was identical for both slices indicating that there was no selective loss of pyridoxal phosphate from the α, β, or γ forms of the soluble enzyme.

Initial steps in soluble enzyme purification procedure (Bertland and Kaplan, 1968) are as follows: (1) water extraction of the frozen hearts; (2) 0–80% $(NH_4)_2SO_4$ precipitation; and (3) dialysis of the redissolved 80% precipitate against water. This is followed by two additional ammonium sulfate precipitations and heat treatment at 55° in the presence of α-ketoglutarate. The soluble enzyme from steps 1, 2, and 3 initially showed three bands of activity on starch gel. A fraction from each step in the purification procedure was taken and stored at 4°. Gel electrophoresis of these fractions 7 days after the start of the purification showed traces of more anionic soluble enzyme proteins, and after 12 days, three to four distinct bands appeared in the soluble enzyme region, migrating more rapidly to the anode than the three original bands. There was also a band which was less anionic than the α band (Figure 1B). The crude extract lost activity upon storage, and could not be followed during this time period. These changes were apparent in all fractions of the purification procedure. Recombination of the first 80% $(NH_4)_2SO_4$ supernatant with the first 80% precipitate did not alter the gel pattern. The appearance of the extra bands

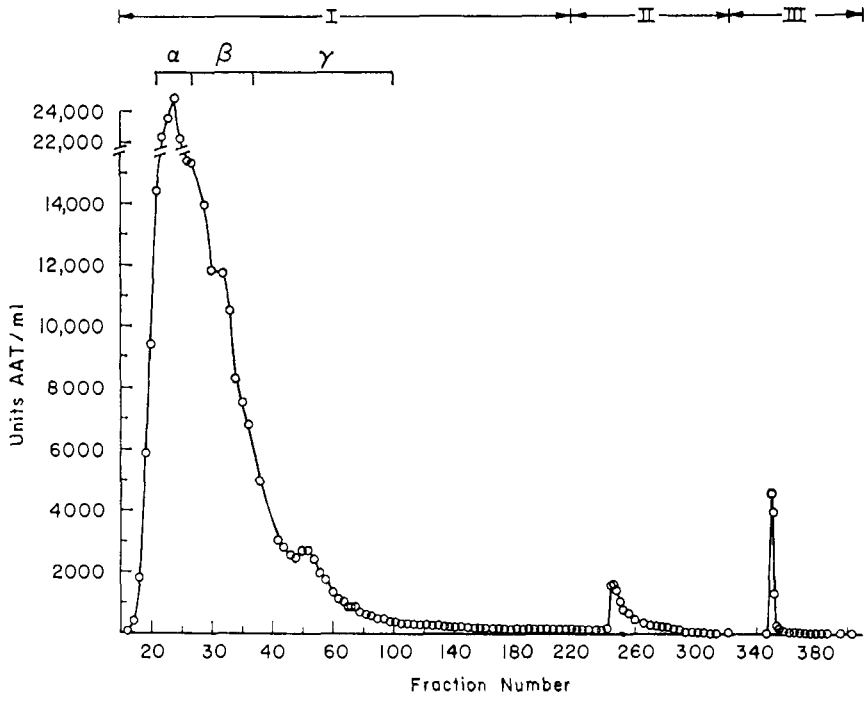

FIGURE 3: Chromatography of chicken heart soluble aspartate aminotransferase on DE-52 cellulose: (I) elution with 0.005 M potassium phosphate buffer (pH 7.5); (II) elution with 0.005 M potassium phosphate buffer (pH 7.5)–0.02 M NaCl; (III) elution with 0.005 M potassium phosphate buffer (pH 7.5)–0.2 M NaCl. See text for other details of the procedure.

of activity in the undialyzed enzyme from step 2 would appear to rule out removal of a small molecule as the cause of the alteration of the electrophoretic pattern. The reason for the appearance of the new bands of activity during the purification procedure is as yet unknown. There was no change in the electrophoretic pattern of the mitochondrial enzyme as the result of the purification procedure utilized.

The purification procedure does, however, remove most of the bands that may have arisen as artifacts. The mitochondrial enzyme is separated by chromatography on CM-11 and the soluble enzymes are then chromatographed on DE-11 in 0.005 M potassium phosphate buffer (pH 7.5). Elution of the column with this buffer removes only the first four or five soluble isozymes (Figure 2, tubes 60–120) with the more anionic bands still bound to the column (Figure 1C, and D). These may be removed by elution of the column with 0.005 M potassium phosphate buffer (pH 7.5)–0.2 M NaCl (Figure 2, tubes 300–400). This material has very low specific activity. Attempts to purify and crystallize these anionic proteins have to date been unseccessful.

The starch gel pattern of the crystalline soluble enzyme is the same as that of the enzyme when eluted from the DE-11 column (Figure 1C). After chromatography on DE-11, no further changes in the electrophoretic pattern of the enzyme occur, even after 2-years storage.

Effect of Small Molecules on the Enzymatic Electrophoretic Pattern. It is possible that the differences in the electrophoretic mobilities of the soluble proteins arise from the binding of a small molecule. The coenzyme pyridoxal phosphate has no effect upon the relative mobilities of the various enzymes. The electrophoretic mobilities of soluble holoenzyme and mitochondrial holoenzyme are identical with those of the apoenzymes, in contrast to the alterations in the electrophoretic mobilities reported for the pig heart soluble enzyme (Banks *et al.*, 1968).

The enzyme used for these experiments was the first 80% ammonium sulfate precipitate which had been dialyzed against water. It contained the mitochondrial enzyme and five to six bands of soluble enzyme activity. The enzyme concentration was 0.5 mg/ml. A number of compounds were incubated with the enzyme for three to four hours at room temperature before starch gel electrophoresis. The following compounds had no effect on the gel patterns of either the soluble or mitochondrial enzymes: L-aspartate, ketoglutarate, oxaloacetate, and L-glutamate (all at 1×10^{-2} M); pyridoxal, pyridoxine, cytosine, thymine and ammonium sulfate (all 2×10^{-3} M), and DPNH (4.6×10^{-3} M). The following metal ions were also found not to effect the electrophoretic pattern in concentrations of 1.66×10^{-3} M: Ca^{2+}, Mg^{+}, Mn^{2+}, Fe^{3+}, and Ni^{3+}. The gel pattern mobility to the anode of both the soluble and mitochondrial enzymes. Ag^{2+} inactivates the mitochondrial enzyme and almost completely inactivates the soluble one. EDTA at a concentration of 0.17 M and 8.44×10^{-4} M 8-hydroxyquinoline have no effect on the gel pattern. Mercaptoethanol (0.1 M) also has no effect on the gel pattern. The crystalline enzyme (Figure 1C), containing five to six bands of activity, was tested for the presence of carbohydrates by the method of Dubois *et al.* (1956) using glucose as a standard. The carbohydrate content of the enzyme was 0.4% or 0.3 mole of hexose per subunit, assuming 50,000 as the molecular weight of the subunit.

Separation of the Multiple Forms of Chicken Heart Soluble Enzyme. Multiple forms of the soluble enzyme were separated by ion-exchange chromatography on Whatman DE-52 resin (Figure 3). Other resins did not result in such a high

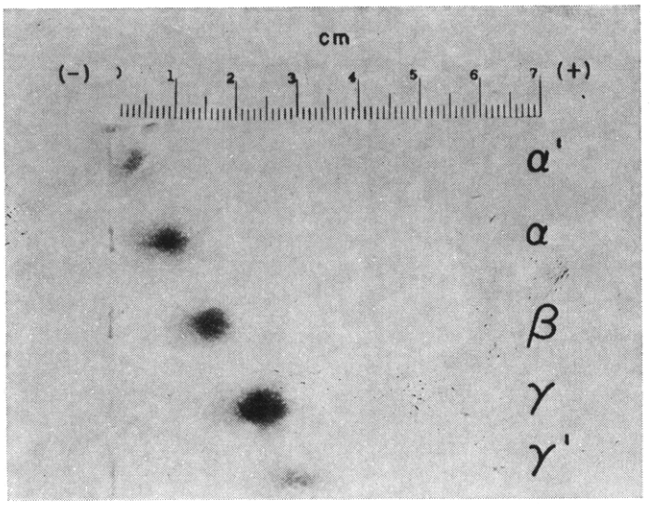

FIGURE 4: Starch gel electrophoretic pattern of the final fractions from chromatography of soluble enzyme on DE-52. α' and γ' are produced during the purification of the enzyme. α, β, and γ are the three proteins in fresh chicken heart extracts.

degree of separation. The DE-52 was equilibrated with 0.005 M potassium phosphate buffer (pH 7.5); the column dimensions were 1.5 × 36 cm. Soluble enzyme (110 mg) was dialyzed extensively against 0.005 M potassium phosphate buffer (pH 7.5). The enzyme was applied to the column in a volume of 10 ml. The column was eluted with 0.005 M potassium phosphate buffer (pH 7.5) at a flow rate of 17 ml/hr. Fractions (2 ml) were collected. The front part of the activity peak contained the α protein, the back part of the peak contained the β proteins, and the γ protein was eluted at the trailing edge of the peak. When the level of activity coming off the column had decreased to 100 μ/ml, the column was eluted successively with 0.005 M potassium phosphate buffer (pH 7.5)–0.02 M NaCl and 0.005 M potassium phosphate buffer (pH 7.5)–0.2 M NaCl. These two buffers eluted small amounts of activity, which contained a mixture of all the active bands and possible may correspond to the fraction V of Bossa *et al.* (1968). The specific activity of these few fractions was lower than that of the first fractions obtained; no further attempt was made to characterize these proteins. Elution with higher ionic strength buffers did not remove additional activity. Much of the activity applied (75%) was recovered and judicious combination of the column fractions made it possible to obtain α, β, and γ proteins free of cross contamination (Figure 4). The separated proteins were stable, and there was no evidence of interconversion even after 18-months storage at 4°.

Amino Acid Composition. Table II gives the compositions of soluble enzyme, bands α, β, and γ as well as that of the mitochondrial enzyme. The amino acid compositions of the α, β, and γ bands are quite similar, if not identical. In contrast, there are significant differences between the compositions of the soluble and mitochondrial enzymes, especially in lysine, histidine, aspartic acid, tryptophan, tyrosine, and valine moieties. All the soluble forms had 9 residues of tryptophan per 50,000.

Molecular Weights. The possibility exists that the bands observed on gel electrophoresis result from aggregation of the enzyme molecules into several molecular weight levels. All the soluble enzymatic bands were eluted from Sephadex G-100 as symmetrical peaks, and electrophoresis of fractions from a G-100 column indicates no separation of the various forms by Sephadex.

Ultracentrifugation of mitochondrial enzyme at a protein concentration of 8.6 mg/ml in 0.05 M potassium phosphate buffer (pH 7.5) yields a sedimentation coefficient of 5.43 S, which is similar to that of the soluble enzyme which has an $s_{20,w}$ of 5.5 S at zero concentration (Bertland and Kaplan, 1968). The molecular weights of both the mitochondrial and soluble enzymes appear to be similar and are in the area of 95,000–100,000.

Circular Dichroism of Chicken Heart Aspartate Aminotransferases. Figure 5 shows the circular dichroism spectra of unfractionated soluble enzyme and the separated α, β, and γ proteins. The shape of the curves for the four proteins is similar. The α, β, and γ forms show slight negative bands at 208 and 222 mμ which are characteristic of the α-helical structure (Holzworth and Doty, 1965). However, there seems to be a high degree of β structure in these proteins, especially

TABLE II: Amino Acid Compositions of Mitochondrial Aspartate Aminotransferase, Unfractionated Soluble Aspartate Aminotransferase, and the Three Proteins of Soluble Aspartate Aminotransferase.

	Amino Acid Residues to the Nearest Integer Calculated for a Mol Wt of 50,000				
			Soluble Enzyme Proteins		
Amino Acid[a]	Mitochondrial Enzyme	Soluble Enzyme Mixture	α	β	γ
Lysine	31	22	22	24	22
Histidine	13	8	7	7	8
Arginine	22	24	24	25	24
Aspartate	50	56	56	55	56
Threonine	27	30	32	30	30
Serine	29	31	33	31	33
Glutamate	46	48	47	46	47
Proline	20	23	24	24	24
Glycine	39	41	41	41	41
Alanine	43	43	44	42	42
Valine	26	32	31	31	30
Methionine	15	12	12	12	12
Isoleucine	30	24	23	23	23
Leucine	40	41	42	41	40
Tyrosine	17	13	13	13	12
Phenylalanine	24	26	26	25	25
Tryptophan	7	9	9	9	9

[a] The values for serine and threonine were determined by extrapolation to zero time. For determination of isoleucine, leucine, and valine, the 72-hr values were used. All other values were obtained by averaging the values for the 24-, 48-, and 72-hr hydrolysates. The values for amide nitrogen and half-cystine were not determined.

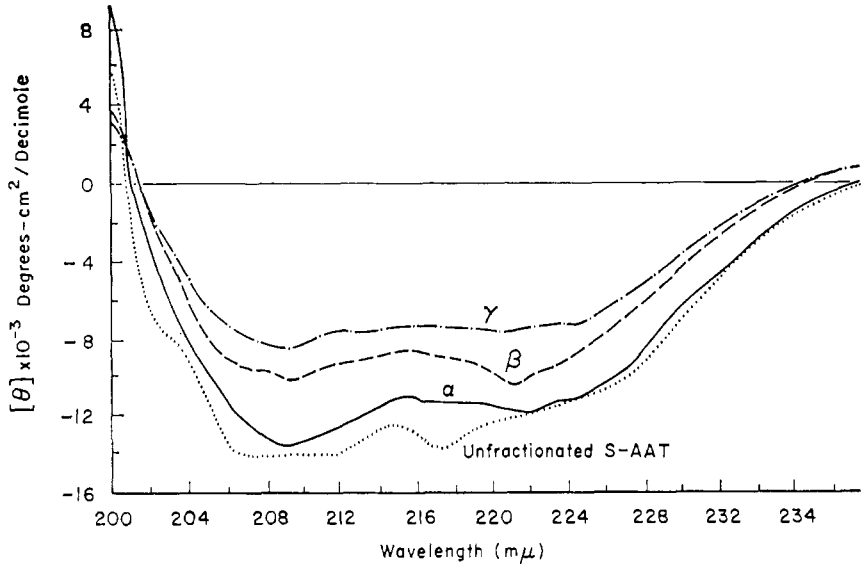

FIGURE 5: Ultraviolet circular dichroism of unfractionated soluble enzyme and the α, β, and γ proteins in 0.01 M Tris-HCl buffer (pH 7.4). The protein concentration was 0.1–0.2 mg/ml. The temperature was 25°.

in the unfractionated mixture which also exhibits a slight negative band at 217–218 mμ. This is characteristic of β structure (Sarkar and Doty, 1966). The molar ellipticities of α, β, and γ decrease in order of increasing mobility toward the anode. The unfractionated soluble enzyme has a greater molar ellipticity than the separated proteins. Such a difference indicates the possibility of interaction between the α, β, and γ proteins in the unfractionated soluble enzyme. It is possible that denaturation occurs during the fractionation procedure, but the specific activities of α, β, and γ are high, and identical with that of the unfractionated mixture (Table III).

The mitochondrial enzyme appears to have an α-helical conformation (Figure 6) as indicated by definite negative dichroic bands at 207 and 223 mμ, and there is a clear separation between them at 215 mμ. The molar ellipticities of the soluble and mitochondrial enzymes are identical at 207 mμ.

Infrared Spectrum of Soluble Holoenzyme. The infrared spectrum of a solid film of soluble enzyme also presents evidence for the existence of β structure in the protein. The maximum is at 1652 cm^{-1} which indicates a majority of α-helical structure (Susi *et al.*, 1967; Timasheff *et al.*, 1967). There are, however, distinct peaks at 1637 and 1685 cm^{-1} which are attributed to the presence of antiparallel chain-pleated sheet structure.

Kinetic Properties. In view of the differences in conformation of the α, β, and γ proteins, it was expected that differences would also be found in their kinetic properties. Apparent K_m's and V_{max}'s of the soluble α, β, and γ proteins, as well as the mitochondrial enzyme were determined and are given in Table III. The apparent K_m for α-ketoglutarate was determined by maintaining the aspartate concentration constant at 33.3 mM, and varying the ketoglutarate concentration between 0.0133 and 0.33 mM for the soluble enzymes, and between 0.2 and 6.67 mM for the mitochondrial enzyme. The apparent K_m for aspartate was determined by keeping the α-ketoglutarate concentration constant at 6.67 mM, and then varying the aspartate concentration between 0.667 and 33.3 mM for the soluble enzymes, and between 0.0667 and 6.67 mM for the mitochondrial enzyme. Enzyme concentrations in these experiments were 3 μg/ml; the buffer used was 0.1 M Tris-HCl (pH 7.4).

The apparent K_m's and apparent maximal velocities of the three subforms were identical with that found with the unfractionated soluble enzyme. The mitochondrial enzyme has an increased affinity for aspartate and much less affinity for ketoglutarate than the soluble enzyme. The apparent maximal velocity of the mitochondrial enzyme is also lower than that of the soluble one.

Heat Stability of the Chicken Heart Aminotransferases. Stabilities of the unfractionated soluble holoenzyme, α-soluble holoenzyme, and γ-soluble holoenzyme were compared by heating the enzymes at a protein concentration of 3 μg/ml in 0.1 M Tris-HCl buffer (pH 7.4) at 65°. The activity was measured every 5 min for 30 min. The rate of denaturation of the three samples was first order, and the first-order rate constants were identical (2.3×10^{-2}). α-Soluble apoenzyme and γ-soluble apoenzyme had identical

TABLE III: Kinetic Properties of Chicken Heart Aspartate Aminotransferases at 25°.[a]

Enzyme	Kg (mM)	K'_{Asp} (mM)	V'_{max} (units/mg)
Unfractionated soluble enzyme	0.079	3.33	4680
α-Soluble enzyme	0.078	3.67	4870
β-Soluble enzyme	0.083	4.50	4640
γ-Soluble enzyme	0.081	3.77	4530
Mitochondrial enzyme	1.45	0.55	3130

[a] Kg, ketoglutarate; Asp, aspartate.

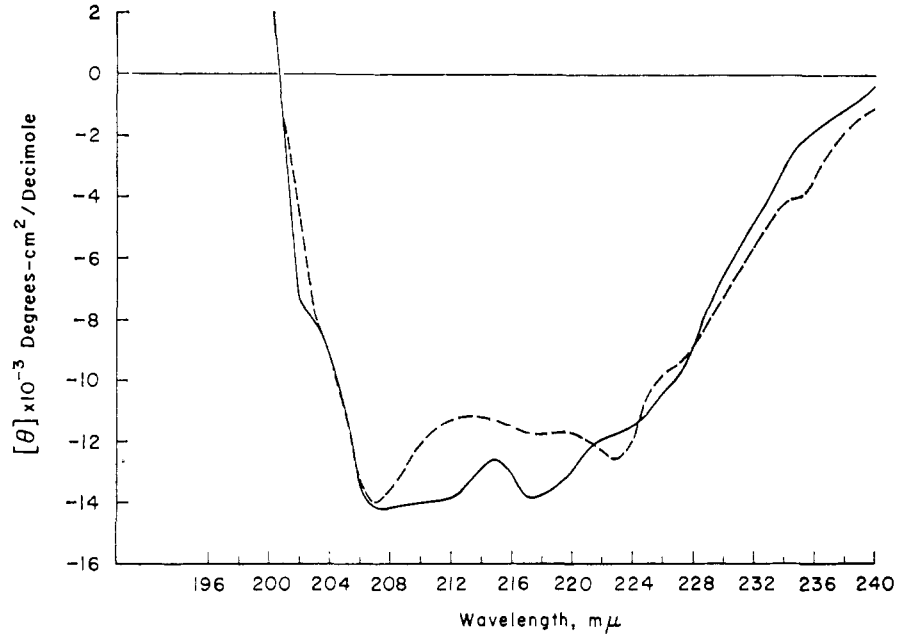

FIGURE 6: Ultraviolet circular dichroism of soluble enzyme (——) and mitochondrial enzyme (- - - -) in 0.01 M Tris-HCl buffer (pH 7.4). The protein concentration was 0.1–0.2 mg/ml. The temperature was 25°.

heat stabilities, although they were less stable than the holoenzymes.

The mitochondrial enzyme was less stable than the soluble enzyme (Table IV) although protection by aspartate, ketoglutarate, phosphate, and pyridoxal phosphate was found to be qualitatively similar to that which has previously been found for the soluble enzyme (Bertland and Kaplan, 1968).

Visible Absorption Spectra. When the catalytically active pyridoxal phosphate is completely removed from soluble enzyme, there still remains a shoulder in the visible absorption spectrum in the area of 330 to 340 mμ (Figure 7, line 1) which corresponds to the absorption maximum of pyridoxamine phosphate. In the soluble holoenzyme (Figure 7, line 2) the 330-mμ region is obscured by the catalytically active pyridoxal phosphate. Addition of α-ketoglutarate to the holoenzyme at pH 5.5, shifts the absorption maximum to 430 mμ, and makes feasible an examination of the 330-mμ region (Figure 7, line 3). Since absorption at 330 is still present after the addition of ketoglutarate to the holoenzyme, the shoulder may be due to the presence of catalytically inactive pyridoxal phosphate which cannot be removed from the enzyme by conventional resolution procedures. The presence of such an entity in pig heart soluble enzyme has been demonstrated by Martinez-Carrion *et al.* (1967).

Attempts were made to isolate the 330-mμ-absorbing component. These attempts were unsuccessful due to the low amount of this component that is bound to the protein. A fluorescence spectrum of soluble apoenzyme was obtained by excitation of the preparation at 340 mμ in 0.1 M potassium phosphate buffer (pH 6.5). The emission peak was quite broad with a maximum at 435 mμ. This corresponds to the broad emission peak with a 430–440-mμ maximum of the soluble holoenzyme. The soluble holoenzyme–L-aspartate complex has a sharp peak with a maximum at 408 mμ and when excited at 334 mμ.

The spectra of the α, β, and γ holoenzyme were determined in 0.05 M potassium phosphate buffer (pH 5.5) and a 100-fold excess of ketoglutarate. Figure 8 shows the difference spectra of the β and γ proteins relative to the α protein. The absorption of the three proteins are nearly identical at 430 mμ, indicating that each has the same amount of active coenzyme. The absorptions of the β and γ proteins are higher than that of the α in the 300–400-mμ range with a slight maximum in the difference spectrum at 305–310 mμ.

There was also a difference in the ultraviolet spectra of the α, β, and γ proteins (Figure 9). The unfractionated

TABLE IV: Heat Stabilities of Chicken Heart Soluble and Mitochondrial Aspartate Aminotransferases.

Incubation Mixture[a]	Soluble Enzyme $t_{1/2}$ (°C)	Mitochondrial Enzyme $t_{1/2}$ (°C)
Apoenzyme alone	42.7	32.5
Apoenzyme plus 1×10^{-2} M α-ketoglutarate	50.9	40.2
Apoenzyme plus 1×10^{-2} M L-aspartate	45.2	36.5
Apoenzyme plus 2.5×10^{-4} M potassium phosphate	56.0	50.0
Holoenzyme	66.0	58.6

[a] The final concentration of enzyme in the incubation mixture was 3 μg/ml of 0.1 M Tris-HCl buffer (pH 7.4). $t_{1/2}$ is defined as the temperature at which one-half of the initial activity is lost after a 10-min incubation.

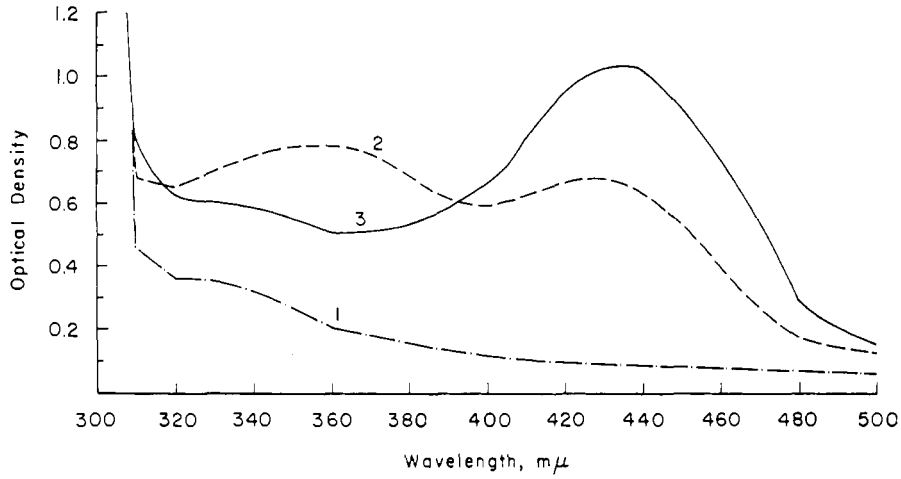

FIGURE 7: Visible absorption spectra of unfractionated soluble aspartate aminotransferase. Protein concentration was 7.4 mg/ml: (1) soluble apoenzymes in deionized water; (2) soluble holoenzyme in 0.05 M potassium phosphate buffer, pH 5.5; (3) soluble holoenzyme in 0.05 M potassium phosphate buffer (pH 5.5)–7.2×10^{-3} M α-ketoglutarate.

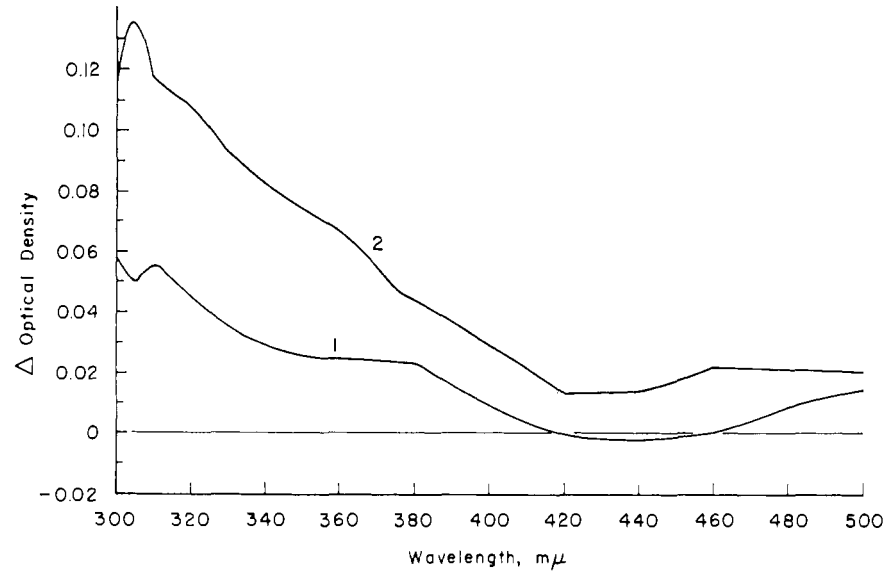

FIGURE 8: Difference spectrum of soluble holoenzyme β and γ proteins relative to the holo-α protein. Protein concentration is 2.00 mg ml in 0.05 M potassium phosphate buffer (pH 5.5)–3.6×10^{-3} M α-ketoglutarate: (1) β protein relative to the α protein; (2) γ protein relative to the α protein.

soluble holoenzyme has the lowest absorption at 250 mμ; whereas the absorption of the separated proteins increases with increasing electrophoretic mobility. The same order is found for the apoproteins, with the unfractionated soluble apoenzyme having a lower absorption than the separated isozymes. Removal of the active coenzyme thus does not alter the relative ultraviolet spectra, although the 250-mμ absorption of the apoenzyme is somewhat lower than that of the holoenzyme. Interaction of the proteins in the unfractionated mixture is again indicated by the lower ultraviolet absorption of the mixture.

Fluorescence Spectra. Fluorescence spectra of the three soluble holoenzyme proteins were obtained by exciting the proteins at 280 mμ in 0.01 M Tris-HCl buffer (pH 7.4) (Figure 10). The three proteins had the same emission maximum at 338 mμ, but the relative quantum yield of fluorescence decreased with the increasing negative charge of the subforms.

Effect of Borohydride. In order to establish that the variation in electrophoretic mobility is not due to differences in covalent binding of the pyridoxal phosphate *at the active site*, the catalytically active pyridoxal phosphate was bound covalently to the lysine residue at the active site by reduction with sodium borohydride. Sodium borohydride (5 mg) was added to 2.4 mg of soluble holoenzyme in 0.5 ml of 0.1 M Tris-HCl buffer (pH 7.4). The reaction continued for 15 min, and the excess sodium borohydride was then removed by dialysis against 0.1 M Tris-HCl (pH 7.4). Starch gel electrophoresis showed the sodium borohydride treated

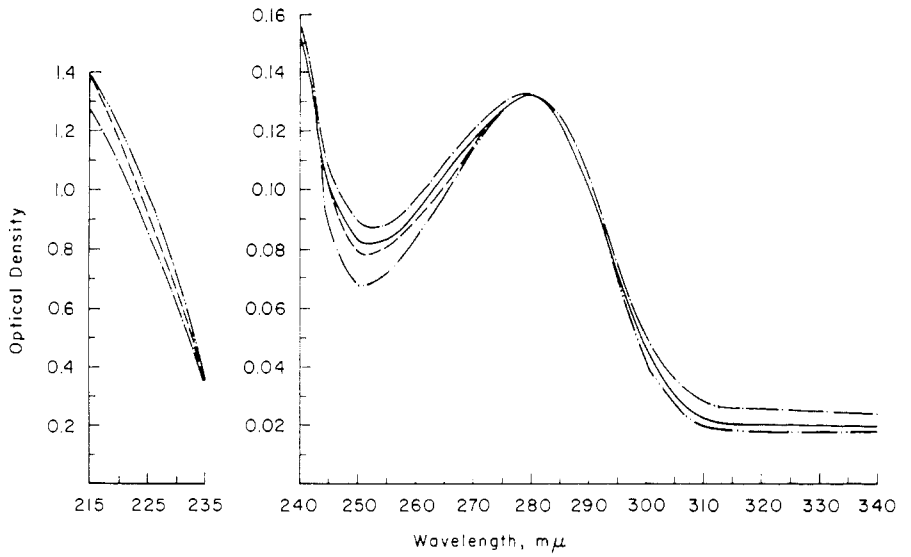

FIGURE 9: Ultraviolet absorption spectra of unfractionated chicken heart soluble enzyme and the α, β, and γ proteins in 0.05 M potassium phosphate buffer (pH 5.5). All samples were diluted to have an optical density of 0.13 at 280 mμ: (— — —) α-soluble enzyme; (——) β-soluble enzyme; (-·-·-·-) γ-soluble enzyme, (-· · ·-· · ·); unfractionated soluble enzyme.

enzyme gave the same mobility and distribution of isozymes on starch gel as the native enzyme. Most (98%) of the activity was lost by this procedure.

The mobilities were found to be altered by covalent binding of pyridoxal phosphate to lysine residues other than the active-site residue. The borohydride procedure was repeated in the presence of a 200-fold excess of pyridoxal phosphate. The starch gel pattern of soluble enzyme modified in this manner showed a decrease in the intensities of the original bands, and the appearance of a more anionic series of proteins similar in appearance to the enzyme eluted at high ionic strength from DE-11 (see Figure 1D).

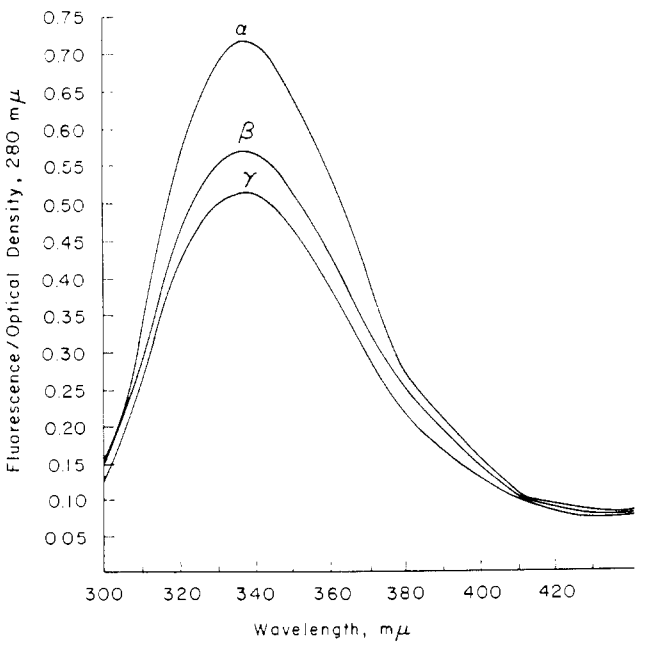

FIGURE 10: Fluorescence emission spectra of α, β, and γ proteins of soluble enzyme. Excitation at 280 mμ. The buffer was 0.01 M Tris-HCl (pH 7.4).

Reactivation of Soluble Apoenzyme from Gd·HCl.[1] If α, β, and γ represent conformational variants of one amino acid sequence, it should be possible to convert one conformation of the enzyme into another by reversible inactivation by a suitable denaturing agent such as Gd·HCl. Soluble apoenzyme was added to a solution of Gd·HCl to give a final concentration of 1 mg/ml in 6 M Gd·HCl in 0.1 M Tris-HCl buffer, pH 7.4. After 15 min, the enzyme was diluted 20-fold into a reactivation mixture. The activity was measured with pyridoxal phosphate at 5-min intervals after dilution. The presence of mercaptoethanol and pyridoxal phosphate was absolutely essential for recovery of enzymatic activity (Figure 11). The holoenzyme also required excess pyridoxal phosphate and mercaptoethanol for reactivation to take place. The effects of anions, pH, and ionic strength are given in Table V. Pyridoxamine phosphate could be substituted for pyridoxal phosphate. The final reactivation mixture was composed of 0.05 M potassium phosphate buffer (pH 7.5), 0.1 M mercaptoethanol, and 5×10^{-5} M pyridoxal phosphate.

Reactivation of the apo- and holoenzymes did not alter the starch gel pattern of these enzymes. Even after 24 hr in 6 M Gd·HCl, the gel pattern of the unfractionated enzyme was identical with that of the control. The α protein and the γ protein were also reversibly denatured in guanidine. No change in their starch gel pattern occurred. The recovery of activity was lower for the reported subforms than for the unfractionated soluble enzyme. Only 10% of the band activity was recovered after inactivation and 20% of the γ-band activity was recovered after denaturation with the guanidine.

[1] The abbreviation used is: Gd·HCl, guanidine hydrochloride.

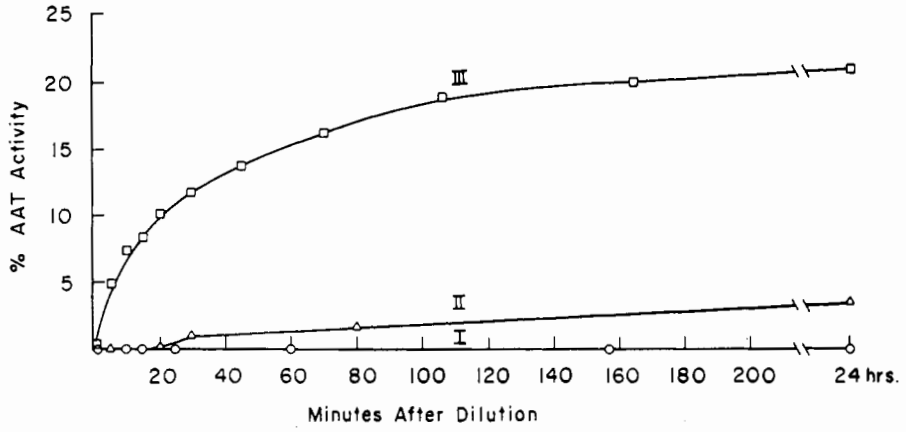

FIGURE 11: Reactivation of soluble apoenzyme from 6 M guanidine. After 15 min in 6 M Gd·HCl, 0.1 ml of the enzyme (1 mg/ml) was added to the following mixtures: (○–○–○) 1.9 ml of 0.1 M Tris-HCl (pH 7.4); (△–△–△) 1.9 ml of 0.1 M Tris-HCl (pH 7.4)–0.1 M 2-mercaptoethanol or ml of 0.1 M Tris-HCl buffer (pH 7.4)—5×10^{-5} M pyridoxal phosphate; (□–□–□) 1.9 ml of 0.1 M Tris-HCl buffer (pH 7.4)–0.1 M 2-mercaptoethanol–5×10^{-5} M pyridoxal phosphate.

Discussion

Comparison of the properties of chicken heart soluble aspartate and mitochondrial aspartate aminotransferases definitely indicates that they are two distinct proteins. The differences in the electrophoretic mobilities of the two proteins may be attributed to the differences in their amino acid compositions. The mitochondrial enzyme is less stable to heat than the soluble enzyme, but it is protected by coenzymes, substrates, and phosphates in a manner similar to that observed with the soluble enzyme. Even though these proteins catalyze the same reaction, they have different affinities for aspartate and α-ketoglutarate.

It was of interest to determine how these variations in the amino acid composition would affect the three-dimensional conformation of the proteins. Examination of the circular dichroic spectra of the two aspartate aminotransferases shows that they have the same molar ellipticities at 208 mμ but that the shapes of the two curves are different. The mitochondrial enzyme contains mainly α-helical structure, whereas the soluble enzyme has regions of β structure as well as some α helix.

Alterations in the amino acid compositions in other isozymes seem to have varying effects on the circular dichroic spectra. Human carbonic anhydrases B and C have marked differences in the degree of ellipticity, although the shapes of the curves are the same in the area of 210–240 mμ (Beychok *et al.*, 1966). The isozymes of peroxidase differ in amino acid composition as well as in the amount of carbohydrate bound to the enzymes (Shannon *et al.*, 1966); nevertheless, their dichroic spectra in the ultraviolet area between 180 and 250 mμ are identical (Strickland *et al.*, 1968).

Microheterogeneity of proteins has been discussed by several authors (Colvin *et al.*, 1954; Haurowitz, 1956; Epstein and Schechter, 1968; Kaplan, 1968). The presence of microheterogeneity in an enzyme could be due to any of the following reasons: genetic variations which result in differences in amino acid composition, molecular weight aggregates, binding of a small molecule, or differences in the conformation of the proteins. The latter hypothesis would account for the fact that one protein sequence could exist in several thermodynamically stable conformations without interconversion (Epstein and Schechter, 1968).

Examination of the electrophoretic pattern of crude extracts of fresh chicken hearts shows the presence of three active proteins. The distribution and number of these proteins changes during the purification; it has as yet not been possible

TABLE V: Reactivation of Soluble Apoaspartate Aminotransferase from 6 M Gd·HCl.

Reactivation Mixture[a]	% Reactivation after 30 min
1. Variation of buffer components	
0.1 M Tris-HCl, pH 7.4	15
0.05 M Potassium phosphate, pH 7.5	43
0.05 M Sodium pyrophosphate, pH 7.5	43
0.05 M Sodium citrate, pH 7.4	32
2. Variation of pH	
0.05 M Potassium phosphate, pH 6.0	14
0.05 M Potassium phosphate, pH 6.5	16
0.05 M Potassium phosphate, pH 7.0	34
0.05 M Potassium phosphate, pH 8.0	29
3. Variation of ionic strength	
0.005 M Potassium phosphate, pH 7.5	41
0.5 M Potassium phosphate, pH 7.5	12
0.05 M Potassium phosphate, pH 7.5–0.2 M NaCl	41
0.05 M Potassium phosphate, pH 7.5–1.0 M NaCl	15

[a] All reactivation mixtures also contained 0.1 M 2-mercaptoethanol and 5×10^{-5} M pyridoxal phosphate. The inactivation mixture contained 1 mg of soluble apoenzyme in 1 ml of 6 M Gd·HCl in 0.1 M Tris-HCl buffer (pH 7.4). After 15 min, 0.1 ml of the inactivation mixture was added to 1.9 ml of the reactivation mixture.

to determine the reason for these changes. Alterations of the electrophoretic pattern in crude extracts of malic dehydrogenase (Thorne, 1968), mushroom tyrosinase (Jolley and Mason, 1965), and xanthine dehydrogenase (Shinoda and Glassman, 1968) have been reported. Removal of the pyridoxal phosphate does not alter the distribution, nor does the binding of substrates and coenzyme analogs. Of the metal ions tested, only copper and silver had any effect on the gel pattern. Since EDTA and 8-hydroxyquinoline did not have any effect on the gel pattern, the reaction with these metals has been attributed to nonspecific reactions with the various forms.

The presence of all three forms in one chicken heart argues stongly against the possibility that the soluble enzyme multiple forms are genetic variants, although the presence of genetic variants of soluble enzyme in herring has been demonstrated by Odense *et al.* (1966). The amino acid composition of the three forms of soluble enzyme are remarkably similar, which would suggest that the differences in electrophoretic mobility do not arise from differences in amino acid composition. A variation of one or two amino acids would probably escape detection by the analytical methods used for this study, and minor variations in amino acid composition can not be excluded. Since amide nitrogen has not been determined, the possibility arises that the differences in electrophoretic mobility arise from differences in amidation of the three proteins. Martinez-Carrion and coworkers (1967) have found, however, no significant difference in the amide content of the three forms of pig heart soluble enzyme.

The three proteins have identical kinetic properties and heat stabilities although they differ in conformation. Since heat denaturation was measured by loss of activity, it may not be a true indication of the changes in the overall structure of the molecule. Kinetic analysis indicates that the differences in conformation, apparently, do not lie in the region of the protein directly involved in the catalytic process.

The three soluble enzymes proteins appear to differ in the three-dimensional structure. Circular dichroic measurements show that the amount of α-helical structure in the soluble enzyme proteins is inversely proportional to the amount of negative charge of the protein. This finding is supported by previous measurements of the optical rotatory dispersion and polarization of fluorescence of the chicken heart soluble enzyme proteins (Kaplan, 1968). The optical rotatory dispersion of the multiple forms of chicken heart mitochondrial malic dehydrogenase indicates that there is a similar correlation between conformation and charge (Kitto *et al.*, 1966).

Martinez-Carrion *et al.* (1967) have reported that the three forms of pig heart soluble enzyme bind different amounts of catalytically inactive pyridoxal phosphate. The visible absorption spectrum and the fluorescence spectrum of the chicken heart apoenzyme indicate the presence of a firmly bound coenzyme, although in much lower quantities than reported in the pig heart enzyme. It may be significant that the pig heart enzyme is purified as a holoenzyme, whereas the chicken heart enzyme is purified as an apoenzyme. The difference in maximum absorption of the β and γ proteins relative to the α protein, does not lie at 340 mμ (which would be expected if the differences in absorption arose only from the binding of different amounts of pyridoxal phosphate) but at lower wavelengths where there is a gradual increase in the absorption maximum at 305–310 mμ. In addition, the ultraviolet spectra show increasing absorbace in the area of 240–250 mμ for the isozymes in the order of their increasing mobility toward the anode.

These differences in the absorption spectra could reflect changes in the environment of the aromatic residues, which may reflect a change of the conformation in the protein (Wetlaufer, 1962). Binding of a small molecule might also cause a change in the absorption spectrum, as has been demonstrated in the binding of iron to conalbumin (Warner and Weber, 1953). The relatively lower quantum yields of tryptophan fluorescence in the more anionic isozymes may indicate that there has been an alteration in the immediate environment of the tryptophan residues, leading to increased quenching of the tryptophan fluorescence. There was no shift in the maximum emission of the tryptophan as would be expected if the alteration in the conformation of the proteins was sufficiently large to increase the exposure of tryptophan to the medium (Teale, 1960; Brand and Witholt, 1967). It should be emphasized that the number of tryptophans in the various soluble enzyme subforms are the same.

The absorption and circular dichroic spectra of the unfractionated soluble enzyme show more of an α-helical structure than any of the separated proteins. Denaturation of the separated subforms in the fractionation procedure cannot be completely ruled out, but on the basis of their individual specific activities, it would seem that no inactivation had occurred. The possibility exists that interaction among the proteins in the unfractionated enzyme might be the explanation of these unique results.

Interconversion of the three soluble enzyme proteins would be possible if the difference between them were solely one of three-dimensional conformation. Our experiments with reversible denaturation from guanidine hydrochloride have not brought about interconversion, but they may shed some light on the factors necessary for the formation of the tertiary structure of the aminotransferase. There is an absolute requirement for both mercaptoethanol and coenzyme for renaturation. Mercaptoethanol is undoubtedly required to prevent the formation of inactive aggregates. Since the assay mixture always contained an excess of pyridoxal phosphate above the amount needed for catalytic activity, the requirement for pyridoxal phosphate in the reactivation mixture implies that the coenzyme is necessary for proper refolding of the enzyme molecule. The native enzyme is stable without the presence of coenzyme, which would indicate that once the tertiary structure is determined, it is possible to remove the coenzyme without marked alterations in that structure. The order of buffer ions in effectiveness for promoting reactivation is phosphate > citrate > Tris. This is the same order of effectiveness as that for protecting the enzyme against heat denaturation. A similar observation has been made for malic dehydrogenase (Chilson *et al.*, 1965).

As a result of the above discussion, we wish to suggest that the differences in electrophoretic mobility of the three forms of chicken heart soluble asparatate aminotransferase arise from differences in the three-dimensional conformation, or from the binding of a small molecule or variation in the amino acid composition which, as yet, has not been detected.

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