BBA 65796

# RECONSTITUTED ASPARTATE AMINOTRANSFERASE PHYSICAL STUDIES

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(Received April 22nd, 1968)

## SUMMARY

Apo-aspartate aminotransferase (L-aspartate:2-oxoglutarate aminotransferase, EC 2.6.1.1) from pig heart can be recombined with pyridoxal 5-phosphate to form reconstituted species with properties indistinguishable from those of the original enzyme. Two distinct types of enzyme properties were investigated in these studies. (1) Macroscopic size of the protein, and (2) interaction of the cofactor with the protein binding site. The results of the sedimentation and gel filtration studies reveal that the two enzymes (native and reconstituted) are virtually identical in terms of sedimentation coefficient ($s_{20,w}$ 5.4) and molecular weight (mol.wt. 90 000). The interaction of the cofactor pyridoxamine 5-phosphate with the enzyme was investigated by means of fluorescence spectroscopy, since it was found that the luminescence properties of the cofactor are sensitive to variations in the configuration of the catalytic site.

The results of the fluorometric experiments indicate that the native and the reconstituted enzymes can be resolved into apoenzyme and cofactor under similar experimental conditions.

## INTRODUCTION

The physical properties of the enzyme aspartate aminotransferase (L-aspartate: 2-oxoglutarate aminotransferase, EC 2.6.1.1) have been extensively investigated by several laboratories[1-3]. It is well established that this enzyme, which contains two molecules of pyridoxal 5-phosphate per 100 000 mol.wt., can be resolved and reconstituted by the addition of pyridoxal 5-phosphate[4-6]. Although the kinetics of reactivation have been analyzed[7], the physical properties of the reconstituted species were not examined as extensively as those of the native enzyme. In the present paper an attempt is made to characterize the reconstituted species with respect to their physical properties. These studies demonstrate similarities among the native and reconstituted species.

* National Science Foundation Predoctoral Trainee.

## EXPERIMENTAL

### *Methods*

Fluorescence spectra were recorded on a spectrofluorimeter designed in our laboratory. Radiation from a 150-W xenon lamp (Hanovia) was passed through a 500-mm Bausch and Lomb monochromator (blazed at 300 mμ, dispersion 3.3 mμ/mm) and focused onto the cell. The fluorescence emission was observed at right angles to the exciting beam using a 500-mm Bausch and Lomb monochromator (dispersion 3.3 mμ/mm) and detected by an EMI 6256 S photomultiplier tube. The signal from the photomultiplier tube was amplified by a recording photometer (Model 15, Pacific Instruments) and the amplified signal fed to the Y axis of a Moseley X-Y recorder (Model 135 AM) the X axis of which was coupled to the wavelength drive of the analyzing monochromator.

Calibration of the exciting light source was carried out with solutions of rhodamine B in ethyleneglycol or 1-dimethylaminonaphthalene-5-sulphonate in 0.1 M $NaHCO_3$ as fluorescence screens[8]. The detector system was calibrated according to the method of WHITE, HO AND WEIMER[9].

Fluorescence yields of the proteins were determined by integrating their emission spectra using L-tryptophan in water as standard of quantum yield $Q = 0.20$. Quantum yields of vitamin $B_6$ derivatives were determined according to the method of PARKER AND REES[10], with standards of known quantum yields (salicylic acid and quinine sulfate)[11].

Polarization of fluorescence measurements were performed in a photometer designed in our laboratory[12]. Illumination was provided by a xenon lamp (200 W) with wavelengths selected by a quartz prism monochromator (Schoeffel, QPM, 30 S). The incident light was polarized by a Glan–Thompson prism (aperture 12 mm × 12 mm, Crystal Optics, Chicago) and focused onto the thermostated cell. An identical arrangement mounted at right angles to the excitation beam was used to select polarized light along V and H, vertical and horizontal axes, respectively. The emitted light was filtered through a Corning glass filter (CS-0-52) which transmits light at wavelengths longer than 340 mμ. The band width for excitation was 5 mμ in the region 250–310 mμ. An analysis of the various sources of error shows that this device is capable of measuring degree of polarization of fluorescence values to an accuracy of 1% for polarization values greater than 0.1.

Absorbance measurements were performed in a Beckman DU spectrophotometer and protein concentrations were determined by the procedure of LOWRY *et al.*[13].

Sedimentation velocity experiments were conducted in the Spinco Model E analytical ultracentrifuge at constant temperatures in the range 20–22°. For sedimentation velocity studies, the ultracentrifuge was operated at 56 100 rev./min and the sedimentation constants corrected for the density and viscosity of water at 20°. Light scattering measurements were performed in a Brice–Phoenix light scattering photometer. The instrument was calibrated with an opal glass furnished by the manufacturer. Refractive index increments were determined at 25° with a Brice–Phoenix differential refractometer calibrated with solutions of KCl of known concentrations.

### *Materials*

*Preparation of the enzyme.* The enzyme aspartate aminotransferase was purified

according to the procedure of SIZER AND JENKINS[14]. This preparation at pH 8.5 exhibited a symmetrical absorption band at 362 mμ, indicating that the enzyme was in the pyridoxal form. This preparation was purified further according to the method of MARTINEZ-CARRION *et al.*[2]. After chromatography through carboxymethyl-Sephadex C-50, 4 cytoplasmic subforms were collected from the column and the fractions of greater specific activity (Fractions III and IV) were pooled and precipitated with ammonium sulphate and dialyzed against 0.1 M phosphate buffer (pH 7.4). This preparation had a specific activity of 115, expressed as μmoles of oxaloacetate produced per min per mg of protein when assayed according to MARTINEZ-CARRION *et al.*[2]. The light scattering studies were carried out using enzyme prepared according to SIZER AND JENKINS[14]. The enzyme (Fractions III and IV) prepared according to MARTINEZ-CARRION *et al.* was used throughout the remainder of the studies.

*Resolution of the enzyme.* The enzyme was resolved into apoenzyme and cofactor according to the method of SCARDI *et al.*[6]. The resulting apoenzyme showed very little residual activity (1%) and no absorption was detected over the spectral range 320–360 mμ at a protein concentration of 1.5 mg per ml.

*Reconstitution of the enzyme.* The apoenzyme at a concentration of 10 mg per ml in 0.05 M phosphate buffer (pH 7.4) was allowed to react with excess pyridoxal 5-phosphate (20 moles of pyridoxal 5-phosphate per mole of enzyme) at 4° for 24 h. Then the reconstituted enzyme was freed from pyridoxal 5-phosphate which is not specifically bound to the protein by dialysis against 5 changes of 0.1 M phosphate buffer (pH 7.4) at 4°, followed by filtration through a Sephadex G-25 column, (25 cm × 1 cm) equilibrated with 0.1 M phosphate buffer (pH 7.4). The reconstituted species prepared by the above described procedure, showed an excellent recovery of the original activity (95–100%) when assayed for enzymatic activity according to the methods of SIZER AND JENKINS[14], and MARTINEZ-CARRION *et al.*[2]. The steady state kinetic analysis was carried out essentially as described by VELICK AND VAVRA[15]. The graphical treatment developed by these authors was used to determine the $K_m$ values for α-ketoglutarate and L-aspartate at 25°. The reagents pyridoxal 5-phosphate, pyridoxamine 5-phosphate, α-ketoglutarate, L-aspartate and NADH were purchased from Sigma, sodium borohydride from Metal Hydrides Inc. and the enzyme malate dehydrogenase from Worthington. The proteins ribonuclease, bovine serum albumin, bovine γ-globulin, and alcohol dehydrogenase were purchased from Sigma.

## RESULTS

In an attempt to compare the firmness of binding of pyridoxamine 5-phosphate to the catalytic site of native and reconstituted aspartate aminotransferase, the dissociation of the enzyme into apoenzyme and pyridoxamine 5-phosphate was followed by fluorometric technique. To this end, the enzyme (pyridoxal form) at a concentration of 1 mg/ml in 0.05 M phosphate buffer (pH 6.8) was converted to the pyridoxamine form by the addition of L-aspartate (20 μmoles/mg of enzyme). Then the mixture was incubated at 25° for 30 min and the fluorescence spectrum was recorded over the range 340–460 mμ, (exciting wavelength 330 mμ). Under these experimental conditions, the fluorescence emitted by the pyridoxamine 5-phosphate residues attached to the enzyme was characterized by a low quantum yield of fluorescence ($Q = 0.01$). When the pH of the solution was brought to pH 4.8 by addition of $KH_2PO_4$,

to a final concentration of 0.5 M, the dissociation of the enzyme into apoenzyme and pyridoxamine 5-phosphate was accompanied by a substantial increase in the fluorescence intensity emitted at 390 m$\mu$ (Fig. 1). The process was completed within 60 min and the emission spectrum obtained after completion of the reaction displayed the characteristic features of free pyridoxamine 5-phosphate in solution[17]. The change in fluorescence intensity as a function of time, which is essentially due to the release of pyridoxamine 5-phosphate from the enzyme, can be adequately described by the expression:

$$\ln (F_{max} - F_t) = K_{obs} \times t + C \quad (1)$$

Where $K_{obs}$ is the observed first-order rate constant for the removal of pyridoxamine 5-phosphate, $F_{max}$ is the maximum fluorescence intensity value reached after completion of the reaction, and $F_t$ is the fluorescence intensity at time $t$. In view of the facility with which the kinetics of resolution could be measured, it was of interest to examine whether the resolution of the reconstituted enzyme followed a similar kinetic

TABLE I

RESOLUTION AND RECONSTITUTION OF ASPARTATE AMINOTRANSFERASE (PYRIDOXAMINE 5-PHOSPHATE FORM)

| *Sample* | *Rate constant** ($min^{-1}$) | *Activity after*** | |
|---|---|---|---|
| | | *Resolution and dialysis (%)* | *Reconstitution with pyridoxamine 5-phosphate (%)* |
| Native | 0.039 | 1 | 90 |
| Reconstituted | 0.039 | 1 | 85 |

* Rate constant for resolution calculated according to Eqn. 1.

** Activity assays were performed according to MARTINEZ-CARRION *et al.*[2]. The final concentration of enzyme in the reaction mixture was $1.5 \cdot 10^{-9}$ M (assuming a molecular weight of 90 000).

pattern when analyzed by fluorescence spectroscopy. This expectation was fulfilled since the kinetics of resolution of the reconstituted enzyme was found to be identical to that of the native enzyme. The results of these experiments are summarized in Fig. 1 and Table I. The apoenzyme prepared by the above described procedure could be easily reconstituted by addition of pyridoxamine 5-phosphate. To this end, the protein solutions studied by fluorescence spectroscopy were dialyzed against 0.05 M sodium bicarbonate buffer (pH 8.2) at 4°. Then, a reconstitution mixture (3 ml) containing 0.5 mg of apoenzyme per ml and 20-fold molar excess of pyridoxamine 5-phosphate was incubated at 30° for 1 h. The course of reactivation was followed by activity measurements and restoration of approx. 90% of the specific activity of the original enzyme was obtained. It should be noted that a similar recovery in the reactivation of the apoenzyme prepared by several methods has been reported in the literature[7]. The preceding experiments clearly demonstrate that the experimental conditions chosen for resolution of both native and reconstituted enzymes must be

sufficient to insure the cleavage of the electrostatic bonds linking the cofactor to the enzyme. Furthermore, the remarkable similarity of the rate constants describing the release of pyridoxamine 5-phosphate into the solution suggests that the magnitude of the forces holding the cofactor to the active site of either native or reconstituted aspartate aminotransferase are virtually identical.

*Reduction with sodium borohydride*

A series of experiments on borohydride-treated enzymes provided additional information about the degree of exposure of the pyridoxal residues and about the specific interaction with amino acid residues of the enzyme. It is well established that sodium borohydride reduces the azomethine linkage between the formyl group of

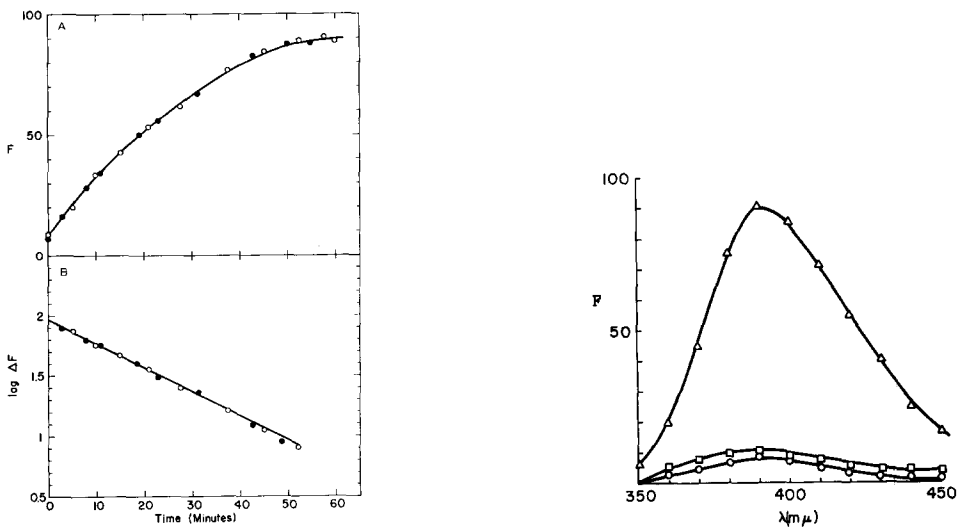



Fig. 1. A. Increase in fluorescence intensity at 390 mμ (excitation wavelength, 330 mμ) that follows the release of pyridoxamine 5-phosphate from the enzyme aspartate aminotransferase in 0.5 M $H_2KPO_4$ (pH 4.8) at 25°. Results obtained with native and reconstituted enzyme are represented by open and closed circles, respectively. Experiments conducted at a protein concentration of 1 mg per ml. B. Plot of $\log \Delta F$ (fluorescence increase at 390 mμ) as a function of time for the resolution of native and reconstituted aminotransferase.

Fig. 2. Corrected emission spectra for pyridoxamine 5-phosphate (△), native (□) and reconstituted aminotransferase (○). The samples of enzyme were reduced with sodium borohydride and dialyzed against 0.1 M phosphate buffer (pH 7.4). The absorbance of the samples at the exciting wavelength (330 mμ) was 0.1 for 1-cm cuvettes. Areas beneath the curves are proportional to the fluorescence quantum yield.

pyridoxal 5-phosphate and an ε-amino group of the lysyl residue in the protein[16]. This reaction inactivates the enzyme and causes an immediate change in the absorption spectrum. Thus the 430 mμ (pH 5) absorbance disappears and there is a corresponding increase in the absorption at 330 mμ. Furthermore, the emission spectrum due to the pyridoxyl residues of the reduced enzyme is characterized by an abnormally low fluorescence yield[17]. Treatment of native and reconstituted aspartate aminotransferase with sodium borohydride at pH 5.6, in order to reduce the linkage of pyridoxyl 5-phosphate to the protein was carried out according to the procedure of HUGHES, JENKINS

AND FISCHER[16]. The reduced samples were dialyzed against 0.1 M phosphate buffer (pH 7.4) and their fluorescence spectra were compared to that of free pyridoxamine 5-phosphate in solution. As shown in Fig. 2 the pyridoxyl residues bound to the active sites of either native or reconstituted aspartate aminotransferase are characterized by an abnormally low fluorescence yield ($Q = 0.01$) when compared to free pyridoxamine 5-phosphate in solution. This unique property of the pyridoxyl groups of aspartate aminotransferase has been attributed to strong interactions with amino acid residues of the protein, since the fluorescence emitted by pyridoxyl chromophores bound to several proteins shows a quantum yield very close to that of free pyridoxamine 5-phosphate in solution[17]. The origin of the quenching processes responsible for the low fluorescence yield of reduced aspartate aminotransferase must be sought in the environment of the pyridoxyl groups, that is, the structures surrounding these chromophores when they are firmly bound to the enzyme.

*Emission and polarization spectra*

In order to obtain further insight into the structural changes produced by resolution and reconstitution of the original enzyme, the fluorescence properties of native, resolved, and reconstituted enzyme were investigated at 25° in phosphate buffer solution. The enzyme aspartate aminotransferase shows a maximum of emission at

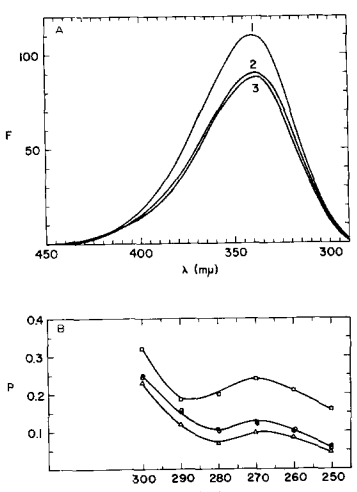



Fig. 3. A. Fluorescence spectra of apoenzyme (1) native (2) and reconstituted aminotransferase (3) excited at 280 mμ in 0.1 M phosphate buffer (pH 7.4). $A = 0.1$ at the exciting wavelength. B. Polarization of fluorescence spectra of apoenzyme (△), native (●) and reconstituted aminotransferase (○) at a protein concentration of 1 mg/ml in phosphate buffer at 25°. Polarization of L-tryptophan ($10^{-4}$ M) in 95% glycerol at 25° (□).

336 mμ; the shape as well as the band position of the emission spectra are insensitive to variations in the exciting wavelength over the spectral range 270–300 mμ. As shown in Fig. 3, a comparison of the emission spectra of native and reconstituted species, reveals a complete coincidence over the spectral region examined. In addition, the quantum yield of fluorescence of the reconstituted species is practically indistinguisha-

ble from that of the native enzyme ($Q = 0.08$). Although the resolved enzyme (apoenzyme) exhibits a $Q$ value slightly larger than that of the native enzyme ($Q = 0.10$), its maximum of emission (336–338 mμ) is very close to that of the active species. Additional information on the perturbation of tryptophan emission induced by conformation changes of the protein was obtained from polarization spectroscopy. The polarization of fluorescence spectra of the proteins in 0.1 M phosphate buffer (pH 7.4) at 25° together with the polarization of fluorescence spectrum of free tryptophan in 95% glycerol at 25° are shown in Fig. 3. It is immediately apparent that the polarization values obtained for the proteins are smaller at all exciting wavelengths than those corresponding to free tryptophan in glycerol at room temperature or in propyleneglycol at 77°K (ref. 18). The overall decrease in the polarization of fluorescence of the proteins as compared to free tryptophan in a rigid medium, can be explained in terms of either energy transfer among tryptophyl residues or rotation of the emission oscillators during the brief lifetime of the excited state, since it is well established that both mechanisms would lead to a decrease in the observed polarization of fluorescence[19].

If the assumption is made that the polarization of fluorescence of proteins is only influenced by changes in local freedom of rotation of the tryptophyl chromophores, then the uniform decrease in the polarization of fluorescence of the resolved enzyme as compared to that of the active species can be ascribed to a loss in rigidity attendant on the removal of the prosthetic group (pyridoxal 5-phosphate). This interpretation is quite in accord with the optical rotatory dispersion studies[20], which also indicate that a conformational change has occurred upon removal of the cofactor from the active site of the enzyme.

*Size of the reconstituted enzyme*

Two independent methods, ultracentrifugation and light scattering, were used to estimate the macromolecular size of reconstituted aspartate aminotransferase. The enzyme aspartate aminotransferase has been reported to have a sedimentation coefficient of 5.4 S, corresponding to a molecular weight of approx. 110 000 (ref. 1). This value remained essentially unaffected by changes in protein concentration at pH 7.5. In the present study it was found that both native and reconstituted aminotransferase have a sedimentation coefficient of approx. 5.4 at a protein concentration of 8 mg per ml in 0.1 M phosphate buffer pH 7.4 (Table II). These sedimentation values were not affected by variation in protein concentration over a range 3–8 mg per ml.

TABLE II

PHYSICAL PROPERTIES OF NATIVE AND RECONSTITUTED AMINOTRANSFERASE

| *Sample* | *Molecular weight** | *Molecular weight*** | *Sedimentation coefficient* | *Fluorescence spectrum* ($\lambda_{max}$) | *Quantum yield* | $K_m$ *(M)* | |
|---|---|---|---|---|---|---|---|
| | | | | | | L-*Aspartate* | α-*Ketoglutarate* |
| Native | 90 000 | 100 000 | 5.4 S | 336 mμ | 0.08 | $2.4 \cdot 10^{-3}$ | $1.3 \cdot 10^{-4}$ |
| Reconstituted | 92 000 | 125 000 | 5.4 S | 336 mμ | 0.08 | $2.3 \cdot 10^{-3}$ | $1.2 \cdot 10^{-4}$ |

* Determined by gel filtration.
** Determined by light scattering.

Although the sedimentation coefficient itself is not a faithful indication of molecular weight, the general similarity of the sedimentation patterns (Fig. 4) suggests that the molecular weight of the enzyme is not affected by the removal and subsequent addition of the cofactor. This hypothesis was strengthened by light scattering measurements designed to evaluate the molecular weight of the reconstituted species. Since the reconstituted enzyme is stable at temperatures below 30°, light scattering measure-

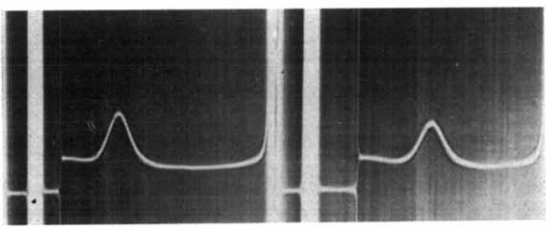
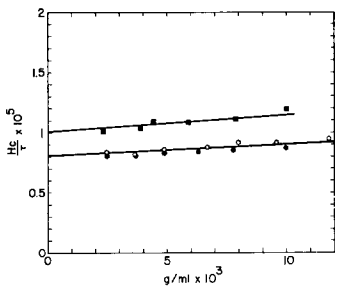


Fig. 4. Sedimentation velocity patterns of native enzyme (left), and reconstituted enzyme (right), at pH 7.4, at 56 100 rev./min, at a phase plate angle of 75°. The photographs were taken 57 and 80 min, respectively, after reaching full speed.

ments can be carried out at room temperature (25°) with little danger of denaturation. The solutions were clarified by repeated direct filtration through type GS millipore filters into the square cells. Scattering ratios were determined at 25° with incident light from a mercury lamp at 546 mμ. In all cases, the light scattering results were analyzed by the equation

$$\frac{Hc}{\tau} = \frac{1}{\bar{M}_w} + 2Bc \qquad (2)$$

where $\tau$ is the turbidity, $c$ the concentration, $\bar{M}_w$ the weight-average molecular weight, $H$ a factor derived from the Rayleigh scattering law, and $B$ the second virial coefficient[21,22]. The results obtained on several samples are shown in Fig. 5. It should be noted that with decreasing concentration of protein, the curve corresponding to the native enzyme approached the ordinate at a level corresponding approximately to a molecular weight of 100 000. The reconstituted enzyme, on the other hand, exhibited a molecular weight (125 000) which is slightly larger than the molecular weight of the native enzyme. This difference is probably due to the presence of aggregated material. It is likely that this contaminant is produced during the resolution of the original enzyme since the molecular weight of the apoenzyme is also larger (125 000) than that of the original enzyme (Fig. 5). Attempts to remove the aggregated material by either centrifugation or chromatography were unsuccessful. Since the ultracentrifugation and light scattering measurements were conducted at protein concentrations larger than 2 mg/ml, it was desirable to compare the behavior of both native and reconstituted species at concentrations approaching those used in the enzymatic assays. To this end, gel filtration studies were carried out with a Sephadex G-200 column (70 cm × 1 cm). The degree to which the column separated macromolecules differing in molecular size was determined by preliminary experiment[5] with proteins of known molecular weight[23,24]. To this end samples of ribonuclease, bovine serum albumin, horse alcohol dehydrogenase, yeast alcohol dehydrogenase, and bovine $\gamma$-globulin were used as



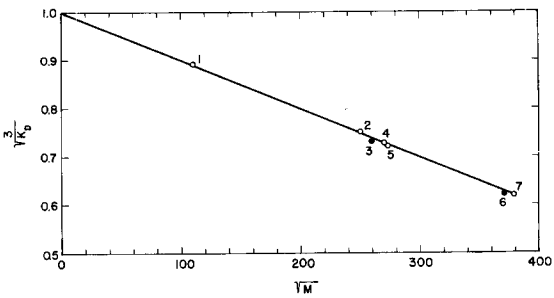




Fig. 5. Plot of $Hc/\tau$ *vs.* concentration, derived from the light scattering measurements of native (■), apoenzyme (○) and reconstituted enzyme (●). Results were obtained in phosphate buffer $I = 0.1$, pH 7.4 at 25°.

Fig. 6. Plot of $\sqrt[3]{K_D}$ *vs.* $\sqrt{M}$ for proteins chromatographed on Sephadex G-200 (70 cm × 1 cm) in 0.1 M phosphate buffer pH 7.4. 1, ribonuclease; 2, bovine serum albumin; 3, horse liver alcohol dehydrogenase; 4, aspartate aminotransferase; 5, reconstituted aspartate aminotransferase; 6, yeast alcohol dehydrogenase; 7, bovine γ-globulin.

standards. When the reconstituted enzyme was filtered through Sephadex G-200 and eluted with 0.1 M phosphate buffer (pH 7.4) it was found that the elution volume of this protein would correspond to a molecular weight of approx. 90 000 (Fig. 6). To check whether the elution profiles of native and reconstituted aspartate aminotransferase were identical, a mixture (0.2 ml) containing 0.05 mg of native enzyme and 0.06 mg of reconstituted enzyme was applied to a Sephadex G-200 column (30 cm × 1 cm) and eluted with 0.1 M phosphate buffer pH 7.4. It was found that the elution profile of the mixture was nearly identical to that displayed by the native enzyme alone. Although the calibration of gel filtration columns according to molecular weight is an empirical method, the remarkable similarity of the elution profiles of both native and reconstituted asparate aminotransferase lend strong support to the contention that differences, if any, in size and shape between these proteins are relatively small.

## DISCUSSION

Several lines of evidence presented in this paper are consistent with the hypothesis that apo-aspartate aminotransferase from pig heart can be recombined with pyridoxal 5-phosphate to form a reconstituted holoenzyme with physical properties similar to those of the original enzyme. Two distinct types of enzyme properties were investigated in this comparative study: (1) Macroscopic size and, (2) binding of the cofactor to the catalytic site. The size of the macromolecules in solution was evaluated by means of gel filtration, light scattering and ultracentrifugation. The results of the sedimentation studies, indicate that the two proteins (native and reconstituted) are virtually identical in terms of sedimentation coefficient. This conclusion was substantiated by gel filtration experiments, which demonstrated that at protein concentrations approaching those normally used in enzymatic assays, the active species (native and reconstituted enzymes) displayed similar elution patterns. The interaction of the cofactor with the binding site of the enzyme was investigated by fluorescence spectroscopy. In this particular case, measurements of fluorescence intensity have provided useful information about changes in the binding of pyridoxamine 5-phos-

phate. In this connection, it is worthy of note that the spectroscopic properties of pyridoxamine 5-phosphate are sensitive to variations in the structure of the catalytic site; therefore the cofactor itself acts as an indicator of events occurring at the level of the catalytic site. This was demonstrated in the resolution of the active aminotransferases, where the increase in fluorescence intensity at 390 mμ reflects the removal of pyridoxamine 5-phosphate under experimental conditions which are known to cause dissociation of the holoenzyme into apoenzyme and cofactor. The remarkable similarity of the rate constants describing the release of pyridoxamine 5-phosphate into the solution suggests that the magnitude of the forces holding the cofactor to the active site of either native or reconstituted aspartate aminotransferase are virtually identical. Another example of the sensitivity of the fluorescence of these compounds to the microenvironment was provided by fluorescence yield measurements of sodium borohydride-reduced enzymes. The reaction of reconstituted aspartate aminotransferase with sodium borohydride inactivated the enzyme and caused immediate changes in the absorption and fluorescence of the cofactor. Furthermore, the emission spectra due to the pyridoxyl residues covalently bound to the enzymes were characterized by an abnormally low fluorescence yield. This unique property of the pyridoxyl residues of aspartate aminotransferase reflects the degree of interaction of these chromophores with amino acids located in the catalytic site of the protein.

ACKNOWLEDGEMENTS

We are indebted to Dr. A. CHERNOFF of the Memorial Research Center, Knoxville for making available the Spinco Ultracentrifuge. This work was supported by a Grant from the Atomic Energy Commission No. AT(40–1)–3553.

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