Dissociation of Aspartate Aminotransferase into Subunits

EFFECT OF LIGANDS UPON THIS DISSOCIATION*

(Received for publication, October 21, 1974, and in revised form, July 11, 1975)

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The quaternary structure of aspartate aminotransferase (EC 2.6.1.1) pig heart cytosolic enzyme, has been studied extensively during the past several years. This problem is of the greatest interest because it is connected with the question of the biological activity of oligomeric proteins and especially with the question of regulation of the catalytic activity of enzymes.

All reports agree that the enzyme is a dimer (molecular weight 93,500 according to the primary structure (1)). It was even postulated that upon dilution it dissociates into catalytically active monomer subunits (2-4). However, conflicting observations have been made by others (5) who report the lack of dissociation induced by dilution. Bertland and Kaplan (6) report that another cytosolic aspartate aminotransferase can dissociate at pH 8 to 9.

In trying to understand an enzymatic mechanism, it is interesting to investigate the relationship between the catalytic activity and the quaternary structure of the enzyme in the presence of substrate. We previously studied the catalytic activity of aspartate aminotransferase at various enzyme concentrations, and differences in the Michaelis constant were observed with L-aspartate (7). The present work is a study of the aggregation state as a function of protein concentration of aspartate aminotransferase alone and in the presence of various substrates and ligands. Molecular sieve chromatography, ultracentrifugation, and fluorescence depolarization measurements have been used.

MATERIALS AND METHODS

Enzyme—The aspartate aminotransferase (EC 2.6.1.1) was purified according to the Jenkins method (6) using succinate instead of maleate; 10^-4 M dithiothreitol was added during the preparation. A further purification on DEAE-Sephadex equilibrated with 0.02 M phosphate buffer, pH 7.4, gave a form contaminated by a very weak 3 form (less than 10%). Some experiments were performed onto very pure a form, prepared according to Martinez-Carrion et al. (9). Apoenzyme free of phosphate was prepared according to Wada and Snell (10, 11).

Concentrations were determined by light absorption at 280 nm using as molar absorptivity 135,000 for the pyridoxal phosphate holoenzyme dimer and 128,000 for the apoenzyme.

Enzyme activities were determined according to Karmen (12) using 20 mM L-aspartate and 2 mM a-ketoglutarate as velocity maximum conditions. A Cary 16-K spectrophotometer was used.

Molecular Sieve Chromatography; Frontal and Zonal Analysis—The exclusion volume of aspartate aminotransferase from Sephadex G-100 is higher than that for a 93,000 daltons molecule (4); therefore, we used Bio-Gel P-150 which is a polyacrylamide gel and does not interact with the enzyme. Frontal analysis was carried out according to Winzor (13); a column (1 x 60 cm) was used and a flow rate of 12 ml/hour was maintained with a LKB peristaltic pump; 0.20 ml fractions were collected, and 10 ml samples of solutions were applied. Their concentration was varied from 5 mM to 2 % M. For initial concentrations higher than 10 mM, the enzyme was detected by fluorescence emission at 340 nm, after 290 nm excitation, with an Amino-Bowman fluorimeter. Smaller concentrations of the enzyme were detected by measuring the activity (in the case of apoenzyme elution, pyridoxal phosphate was added). Detection by either fluorescence or activity has shown similar results. Gel bed volume Vb was measured with blue dextran. According to Winzor (13) and Acker and...
Thompson (14), for a rapidly equilibrating monomer-dimer system, if $V_m$ and $V_c$ are the elution volume, respectively, of the dimer and the monomer and $P$ volume at the centroid of the leading edge of the elution diagram ($\bar{V} = 1/C_p, I = V_c I - 1/C_p \Sigma \Delta C \times V_e$), the weight fraction $a$ of dissociated dimer is:

$$a = \frac{V - V_c}{V_m - V_c}$$

and a relation can be obtained between $C_p$, the plateau concentration of protein (in mole of dimer), $K$, dissociation constant of the dimer, and $a$:

$$K = \frac{a^2}{1 - a} C_p$$

This equation has be fitted for various $K$ values, using a Wang 370 calculator. (The $1/R_p$ value of dimer, monomer, and mixture are determined as $V_m$, $V_c$ or $V$, divided by $V_e$, the elution volume of dextran blue.)

The same experimental conditions were used in frontal analysis, but the volume of the samples was only 0.5 ml instead of 10 ml. In this case, the observations are only qualitative (13).

**Fluorescence Depolarization** — A method analogous to that of Polyakovskiy and Ivanov (2) was used. A fluorescent dye, fluorescein isothiocyanate, was covalently bound to the protein at a ratio 0.1 or 0.2 mole to mole protomer. The dye was incubated with the protein in 0.5 M bicarbonate, pH 8, for a few minutes, and the excess was eliminated by chromatography on Sephadex G-25. Under these conditions, no inactivation was observed.

Fluorescence polarization, $P$, was measured using an apparatus built by Arrio and Rodier and co-workers (15). If the fluorescent dye is rigidly bound to the protein, Perrin's equation is verified and

$$\frac{P}{P_\infty} = \frac{1}{1 + \frac{\tau}{\rho_s \tau_a}} \left( \frac{1}{P} \right)$$

$P_\infty$ — fundamental polarization, $\tau$ — absolute temperature, $\rho_s$ — viscosity, $\tau$ — decay time of the excited state, $\rho_s$ — relaxation time of the protein at the temperature $T$, a plot of $(1/P - 1/\rho_s)$ versus $T/\rho_s$ gives the hydrodynamic volume $v_h$ of the macromolecule. For fluorescein isothiocyanate, this is not true because the dye molecule seems to retain some freedom of intrinsic brownian rotation (16). A good approximation has been given by Weber and Wahl (17): the value of $\rho_s$ or $v_h$ can be calculated if the linear portions of the isotherms are extrapolated to their intersection with the $y$ axis, and the value of $1/P_\infty$ in the formula is replaced by the value of $1/P_\infty$ corresponding to this point intersection for each isotherm. A more accurate calculation has been made by Gottlib and Wahl (18), but the Weber (17) approximation is sufficient to determine relation time of proteins. Any change in this relaxation time induced by dilution is relative to a variation of the aggregation state of this protein, i.e., in the case of aspartate aminotransferase a dissociation of the dimer into monomers.

As already pointed out by Churchich (16), the quantum yield of the dye is not modified in the complex with aspartate aminotransferase. Fluorescence lifetime of free and bound fluorescein was measured using a TRW apparatus and a deuterium flash: a value of 5.3 ns was determined in both cases for all the experimental conditions used in the depolarization experiments.

**Ultracentrifugation** — The sedimentation velocity measurements were performed with the Spinco model E analytical centrifuge (Beckman) equipped with an automatic split beam photoelectric scanning absorption optical system. The protein was sedimented in one sector of a 12-mm double sector cell, the second sector contained buffer. The concentration of protein in the double sector cell was measured with 280 nm wavelength light. The coefficient of sedimentation was approximately determined by evaluating the displacement of the maximum of the difference between two successive scanings. Then $s$ and the diffusion constant $D$ were determined with more accuracy by simulating the sedimentation experiments.

**RESULTS**

**Holoenzyme (Pyridoxal Form)** — A preliminary experiment was performed by zonal analysis on Bio-Gel P-150 (at pH 8.3 in triethanolamine buffer). The $1/R_p$ of a 63.5 $\mu M$ solution of enzyme (6 mg/ml) was 1.20; this value was related to a molecular weight of 90,000 according to a calibration curve. When the enzyme was applied to the column immediately after dilution, a 95 nM solution (8.8 $\mu M$) gave a dissymmetrical peak which had a $1/R_p$ value of 1.27. If the enzyme was maintained at this dilution for 4 hours at 20° before application to the column, two peaks, well separated, were obtained. The $1/R_p$ of the first one was 1.20; the second one was 1.40. After this preliminary experiment showed that the holoenzyme could be dissociated by dilution, a frontal analysis was performed to obtain more accurate results and to determine a dissociation constant for the monomer-dimer equilibrium. By this method, a concentration-dependent variation of $V$ or $1/R_p$ was detected (at pH 8.3 in Tris buffer) (Figs. 1 and 2). With concentrated enzyme, a plateau was observed where $1/R_p$ 1.20 corresponds to the dimeric molecule according to calibration and zonal analysis. With increased dilution, another plateau seems to be observed, but the elution volume is too low ($1/R_p = 1.32$) since it corresponds to a molecular weight of 60,000. From the concentration value corresponding to $a = 0.5$, a dissociation constant of the dimer can be deduced that equals 0.18 $\mu M$. Nevertheless, the experimental values do not fit well with the calculated curves when $K = 0.1$ or 0.8 $\mu M$ is assumed.

Fluorescence depolarization of fluorescein isothiocyanate bound to the holoenzyme confirmed the results obtained from molecular sieving. Fig. 3 shows the Perrin plots obtained for different solutions at least 2 hours after dilution. In these experiments, the buffer was triethanolamine at pH 8.3, 0.05 M ionic strength, and containing $10^{-4}$ M dithiothreitol to avoid SH groups oxidation. These curves gave a $\rho_s$ equal to 106 ns for concentrated holoenzyme at 20°, and this value was constant during 16 hours. Rotational relaxation time became

![Fluorescence depolarization of fluorescein isothiocyanate](http://www.jbc.org/)
smaller with dilution, and the decrease was time-dependent. Since the Perrin plot of concentrated and diluted holoenzyme do not extrapolate to the same apparent 1/P_s value for T/η equal to zero (that means that the dye is a little less rigidly bound to the diluted enzyme), there is no linear relation between 1/P and rotational relaxation time so that the kinetics was difficult to study quantitatively. A study of the variations of the fluorescence polarization of a 0.48 μM solution with time shows that the polarization decrease is slow (Fig. 4). Fluorescence depolarization measurements also allow the reversibility of the phenomenon to show. The addition of concentrated dye-free holoenzyme to diluted labeled holoenzyme causes a rapid increase of polarization, showing that the phenomenon observed by diluting the solution is reversible.

The only possible phenomenon that could be correlated to a variation of the concentration for diluted solutions of protein (less than 1 mg/ml) is a change in the aggregation state of the molecules, and since it was proved in our experiments that the dye is not released in the buffer, the observed decrease in fluorescence depolarization and rotational relaxation time can be correlated to a dimer-monomer equilibrium. Hence two independent methods—molecular sieving and fluorescence depolarization—give the same result: the dimeric pyridoxal form of aspartate aminotransferase dissociates into monomers at pH 8.3 when the concentration is lowered from 1 μM to 10 nM. The dissociation is either a slow phenomenon, or it is rapid and the monomer is slowly transformed.

Apoenzyme—As shown by the frontal analysis of molecular sieving experiments, apoenzyme, at pH 8.3 in Tris buffer can dissociate much more easily than holoenzyme (Fig. 5). In a concentration which is 0.4 μM in dimer, one-half of the molecules are dissociated and a dissociation constant equal to 0.8 μM can be deduced, but the calculated curves do not fit well with the experimental values. At 0.2 μM, apoenzyme is almost entirely dissociated, and addition of one pyridoxal phosphate per monomer gives the elution volume of the dimer. The elution volume of the apoenzyme subunit is consistent with a molecular weight of 46,000.

Since apoenzyme can dissociate in the concentration range where the protein absorbance is 0.02 at 280 nm, ultracentrifugation can be used with a scanning method and double sector cells. Some experiments were made to improve the results obtained by the gel filtration method. With dilution, the diffusion coefficient increases as the sedimentation coefficient falls off as well as the s/D ratio (Fig. 6). These results indicate a drop in the average molecular weight, i.e. a dissociation of the apoenzyme is induced by dilution in the same concentration range where the elution volume increases. Seven hours after dilution, a sample of apoenzyme at 0.2 μM gave an s equal to 2.9 x 10^{-13} s and a D equal to 5 ± 0.5 cm²/s, but the activability was only 70%, instead of 95% obtained 1 hour after dilution.

Effect of Substrates—When the aldiminic form of aspartate aminotransferase can react with the substrate L-aspartate, it is turned into the aminic form of the enzyme (330 nm absorption), and intermediate covalent complexes (360, 430, and 350 nm absorption) exist together (19). Using Bio-Gel chromatog-
In dilution experiments of fluorescence depolarization. This last method gives two other informations: the global phenomenon is slow and its reversibility is confirmed. Frontal analysis also shows that the apoenzyme can undergo dissociation, and the results are confirmed by an independant method—ultracentrifugation.

Our results are consistent with all published observations and may explain some apparent contradictions between them. Actually, others (5, 16) who could not detect enzyme dissociation at 3 nM used sucrose in their experiments, and we observed that a reassociation of the monomer is induced by sucrose. There is some discrepancy between our results of zonal chromatography and that of Feliss and Martinez-Carrion (5). These authors affirm they did not observe any dissociation by Sephados G-200 chromatography, but they did not give their experimental conditions and they did not explain why their results are different from that of Banks et al. or Melander (3), so it is difficult to explain this discrepancy.

Our results on the dissociation of the apoenzyme are consistent with that of Banks et al. (4). These authors failed to obtain a stable apoenzyme and could not reactivate the apoenzyme after passage through Sephados G-100 columns. They found indirect evidence that the apoenzyme dissociates into monomer by studying the kinetics of its recombination with coenzyme. They showed that the apoenzyme undergoes a concentration-dependent change from a more to a less reactive species as the concentration is raised. In our experiments apoenzyme prepared without phosphate does not inactivate on Bio-Gel P-150, so that the chromatographic method can be used and gives a direct measure of the apoenzyme dissociation into monomer. The concentration range is the same for the dissociation into subunits observed by us and for the change in reactivity toward the coenzyme. It means that the hypothesis of Banks et al. is justified: the monomeric apoenzyme is more reactive versus pyradoxal or pyridoxamine phosphate than the dimeric form.

The most important result of the present work is that the stability of the dimeric form is in the order, holoenzyme + substrates > holoenzyme > apoenzyme, with about 1 order of magnitude between the dissociation constants. With the succinylated enzyme, Polyanyovsky and Pikhelgas (20) have shown that the dissociation is easier for the apoenzyme than for the holoenzyme. The order of stability of the quaternary structure and that of the tertiary structure does not seem to be the same, since the complexes with inhibitors are less stable than the pyridoxal enzyme form and more stable than the pyridoxamine form (21).

An important problem is the activity of the dissociated enzyme. Is the monomer really active since substrates induce the dimerization? It seems that in very diluted solutions the activity of the enzyme increases (2, 3, 7), and this increase has been attributed to a lowering of $K_m$ values for aminoacids in enzyme monomeric form. If these differences of $K_m$ values are not due to the methods used to determine the activity at high enzyme concentration, it means that the substrate affinity increases when the enzyme is partially dissociated and that the monomer is active. This result is hardly compatible with the substrate-induced association of the monomer into dimer. The only interpretation compatible with all the observations should be an anticooperativity between the two sites of dimer.

**Acknowledgments**—The authors are greatly indebted to Dr. 

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**Fig. 6.** Sedimentation and diffusion coefficients of aspartate aminotransferase as a function of concentration. (at pH 8.5 in 0.05 M Tris buffer). $s$, sedimentation coefficient of holoenzyme; $D$, diffusion constant; $\Delta$, ratio $s/D$; ---, sedimentation coefficient of apo-enzyme.
Rene Cohen for the program used to simulate the ultracentrifugation experiments. We would like to thank Professor J. Yon and G. Némethy for helpful comments and careful reading of the manuscript. Excellent technical assistance of Mme A. Larousse (ultracentrifugation) and Mme J. Carrette (preparation of the enzyme) is very gratefully acknowledged.

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Dissociation of aspartate aminotransferase into subunits. Effect of ligands upon this dissociation.
I Cournil, J M Barba, D Verge and M Arrio-Dupont

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