Mitochondrial Aspartate Aminotransferase-independent Function of the Catalytic Binding Sites*

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The enzyme mitochondrial aspartate aminotransferase from beef liver is a dimer of identical subunits. The enzymatic activity of the resolved enzyme is restored upon addition of the cofactor pyridoxal-5'-phosphate. The binding of 1 molecule of cofactor restores 50% of the original enzymatic activity, whereas the binding of a second molecule of cofactor brings about more than 95% recovery of the catalytic activity. Following addition of 1 mol of pyridoxal-5'-P per dimer, three forms of the enzyme may exist in solution: apoenzyme-2 pyridoxal 5'-phosphate, apoenzyme-1 pyridoxal 5'-phosphate, and apoenzyme. The enzyme species are separated by affinity chromatography and the following distribution was found: apoenzyme-2 pyridoxal 5'-phosphate/apoenzyme-1 pyridoxal 5'-phosphate/apoenzyme, 2/6/2. Similar distribution was observed after reduction with NaBH₄ of the mixture containing apoenzyme and pyridoxal-5'-P at a mixing ratio of 1:1. Fluorometric titrations conducted on samples of apoenzyme and apoenzyme-1 pyridoxal 5'-phosphate reveal that the enzyme species display identical affinity towards the inhibitor 4-pyridoxic-5'-P (Kᵦᵣᵦ = 1.1 × 10⁻⁴ M). It is concluded that the binding of the cofactor to one of the catalytic sites does not affect the affinity of the second site for the inhibitor. These results, obtained by two independent methods, lend strong support to the hypothesis that the two subunits of the enzyme function independently.

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EXPERIMENTAL PROCEDURE

Fluorescence and Absorption Spectroscopy—Fluorescence measurements were performed in a fluorimeter built in our laboratory (3). Calibration of the exciting source (Xenon lamp, 150 watts) and detector system (EMI 6256S photomultiplier) was carried out as described in a previous publication (3). A band width of 1 nm was used in the fluorescence measurements. Absorption spectra were recorded in a Cary model 15 spectrophotometer.

Enzyme Purification—Mitochondrial aspartate aminotransferase from beef liver was prepared by the method described by Morino et al. (4). This preparation after hydroxylapatite chromatography was contaminated with heme-containing proteins. The contaminating material was separated from the enzyme by chromatography on DEAE-Sephadex A-50 (Pharmacia) as described by Martínez-Carrion and Tiemier (5).

The purified enzyme gives one cationic band at pH 7.8 and pH 8.6 when subjected to polyacrylamide gel electrophoresis and cellulose acetate electrophoresis. This is in contrast to the behavior of the cytoplasmic form of the enzyme which gives several bands when analyzed by polyacrylamide gel electrophoresis. Protein concentration was determined by the colorimetric method of Lowry et al. (6) and by absorbance measurements at 280 nm. A sample of apoenzyme at a concentration of 0.5 mg/ml gives an absorbance of 0.72 at 280 nm (1-cm cuvette).

Pyridoxal-5'-P Content—A sample of purified enzyme gives an absorbance ratio A₃₄₆/A₅₅₈ of 8.6 at pH 8 in 0.05 M Tris HCl. The pyridoxal-5'-P content of the enzyme is 2 mol of pyridoxal-5-P per 90,000 g of protein as determined by the method of Wada and Snell (7).

The molar absorbance coefficient of bound pyridoxal-5-P is 8,300 at 355 nm (pH 8).
Enzymatic Assays-They were conducted in the Cary model 15 spectrophotometer using a coupled reaction with malate dehydrogenase (1).

Resolution of Holoenzyme-The holoenzyme was resolved into apoenzyme and cofactor by the method of Scardil et al. (8), and the release of pyridoxamine-5-P was monitored by fluorescence intensity measurements at 390 nm (excitation 330 nm) (9). The resulting apoenzyme was dialyzed in the cold (4°) against two changes of 0.2 M phosphate (pH 5.7) followed by two changes of 0.05 M Tris-HCl (pH 8). The resolved enzyme was passed through a small Sephadex G-25 column (15 x 1 cm) previously equilibrated with 0.05 M Tris-HCl (pH 8). This last step ensures complete removal of phosphate ions adsorbed to the protein. After gel filtration the apoenzyme displayed little residual activity (1%) when assayed for enzymatic activity.

Affinity Chromatography—Derivatization of Sepharose 4B. Washed Sepharose 4B (Pharmacia) was suspended in 50 ml of distilled water and coupled to 1.6-diaminohexane as described by Cuatrecasas (10). After unreacted ligand was thoroughly washed off, pyridoxal-5-P was later attached to this “spacer” group by the method of Ryan and Fottrell (11). The resulting Schiff’s base was reduced by addition of sodium borohydride at 0°. After reduction the Sepharose was washed off with H2O. This procedure gave an unsolubilized derivative of pyridoxal-5-P. The derivatized Sepharose was equilibrated with 0.05 M Tris-HCl (pH 8) and packed in a small column (14 x 1 cm). Fractions eluted from the column were monitored by fluorescence, absorbance, and enzymatic activity measurements. The pyridoxal 5-P content of the samples eluted from the column was determined by a fluorometric method developed in our laboratory (9).

RESULTS

It is well established that the absorption spectra of the cofactor pyridoxal-5-P covalently bound to the enzyme aspar- tate aminotransferase is influenced by changes in the pH of the solution. At pH 5.3, the mitochondrial enzyme shows an absorption maximum at 430 nm, whereas at pH 6.8, the colorless species are characterized by an absorption band centered at 355 nm. It is now generally accepted that the species absorbing at 430 nm have a positive charge on the nitrogen of the ε amino group of lysine residue bound to the enzyme as a Schiff’s base, while the species absorbing at 360 nm have lost the proton of the aldimine linkage.

The mitochondrial aminotransferase exhibits an emission maximum at 335 nm when excited at 280 nm, indicative of fluorescence emitted by tryptophan residues. The band position of the emission spectra as well as the fluorescence quantum yield of the protein remain essentially invariant when the pH of the solution is increased from 5.3 to 8. However, dissociation of the cofactor pyridoxal-5-P from the protein brings about a substantial increase in the protein fluorescence yield, without any change in the band position of the emission spectra. It should be noted that the fluorescence yield of the holoenzyme is restored upon addition of pyridoxal-5-P to the apoprotein. As shown in Fig. 1, the binding of 2 mol of pyridoxal-5-P per mol of apoprotein brings about a decrease in the protein fluorescence yield; the decrease in protein fluorescence yield is strictly proportional to the fraction of cofactor bound. Thus, the binding of 1 mol of pyridoxal-5-P per mol of protein induces a decrease in protein fluorescence yield which is one-half the value observed when the apoenzyme binds 2 mol of pyridoxal-5-P.

The enzymatic activity of the resolved aminotransferase is gradually restored upon addition of increasing concentrations of pyridoxal-5-P. Thus, the binding of 1 molecule of pyri- doxal-5-P restores 50% of the original enzymatic activity, whereas the binding of a 2nd molecule of pyridoxal-5-P brings about more than 90% recovery of catalytic activity. Although the preceding enzymatic results indicate that the two pyri- doxal-5-P binding sites participate in the catalytic process, they do not convey any information about indirect interaction between the protons.

In an attempt to detect cooperative interaction between the binding sites of the enzyme, we decided to investigate whether saturation of the first binding site on the enzyme has any effect on the affinity of the second binding site as a result of a cooperative effect. Accurate measurements of the binding of pyridoxal-5-P to the apotransaminase are difficult to perform in a standard fluorimeter because the dissociation constant of pyridoxal-5-P, which is in the nanomolar range, would imply fluorescence measurements at nanomolar concentrations of protein. Since measurements at very low concentrations of protein can not be performed in our fluorimeter, we decided to study the binding of the inhibitor 4-pyridoxic-5-P and to investigate whether the affinity of this inhibitor for the apoenzyme is influenced by the presence of pyridoxal-5-P molecules covalently bound to the apoprotein.

4-Pyridoxic-5-P is an ideal inhibitor for these studies because its fluorescence yield is not affected by irradiation in the presence of oxygen. Furthermore, its affinity constant when complexed to the apoprotein is in the micromolar range, a concentration that is suitable for fluorescence measurements in any standard equipment. The interaction of 4-pyridoxic-5-P with the apotransaminase induces several changes in the
fluorescence properties of the ligand. As shown in Fig. 2, the fluorescence yield of bound 4-pyridoxic-5-P is lower than the corresponding yield of the free ligand. The quenching effect associated with binding of the inhibitor is used to determine the affinity constant. The fluorescence intensity of free \( F_o \) and bound 4-pyridoxic-5-P \( F_n \) as well as the fluorescence observed \( F \) when both free and bound ligand are in equilibrium \( F \) were used to calculate the fraction \( \alpha_B \) of ligand bound.

\[
\alpha_B = \frac{F - F_o}{F_o - F_n}
\]  

(1)

where \( F_o \) is the actual observed fluorescence value when the ligand is bound to the protein. Maximum quenching (90%) is observed at a molar ratio of apoprotein to inhibitor of approximately 5:1.

The average number of ligand molecules bound per mol of protein \( \delta \) was calculated from points along the titration curve by means of Equation 2

\[
\delta = \frac{\alpha_B (L_o/P_o)}{\alpha_B (L_o/P_o)}
\]  

(2)

where \( (L_o) \) and \( (P_o) \) are the total inhibitor and protein concentrations, respectively. The results of the fluorometric titrations obtained with the apotransaminase yield a straight line when \( \alpha_B / [L] \) is plotted versus \( \delta \). The dissociation constant determined in this manner is \( 1.1 \times 10^{-4} \) M for each pyridoxic-5-P binding site on the protein.

A dissociation constant of similar order of magnitude was obtained from titration experiments conducted on samples of apotransaminase previously saturated with 1 mol of pyridoxal-5-P per dimer. The dissociation constant determined in this manner represents an average value corresponding to several enzyme species differing in their pyridoxic-5-P content.

**Affinity Chromatography**—The variety of enzyme species formed after saturation of the apotransaminase with 1 mol of pyridoxal-5-P per dimer can be separated by chromatography on derivatized Sepharose. It was thought that by a careful examination of the relative distribution of enzyme species in the elution patterns obtained from the chromatographic column, one should be able to detect any marked deviations from the binomial distribution expected for binding of a ligand to independent sites on the protein.

If the binding displays positive cooperativity, one should be able to detect a relative high concentration of enzyme species containing 2 mol of cofactor per dimer (apotransaminase-2PLP). If, on the other hand, the binding is characterized by negative cooperativity, the elution pattern should consist of a major component (apotransaminase-1PLP) and negligible amounts of the enzyme species (apotransaminase-2PLP) and apotransaminase.

Prior to the affinity chromatography experiments, a sample of apotransaminase at a concentration of 0.2 mg/ml was incubated with pyridoxal-5-P at a molar ratio of 1:1 in 0.05 M Tris-HCl (pH 8) for 1 hour at 25\(^\circ\). The sample (1 ml) was then applied to a column of Sepharose derivatized with N'-(alpha-aminohexyl)-PMP and equilibrated with 0.05 M Tris-HCl (pH 8).

Fig. 3C shows the distribution of the protein species when

\( ^* \) The abbreviations used are: PLP, pyridoxal 5-phosphate; PMP, pyridoxamine 5-phosphate.
the fractions eluted from the column were monitored by fluorescence and enzymatic assay measurements. It is clear that the column separates three different fractions. The protein eluted at pH 8 displays an elution pattern identical with the holoenzyme run under similar conditions (Fig. 3A). The enzymatic activity of this protein is not increased upon further addition of PLP, indicating that it is already saturated with the cofactor. The protein eluted from the column with 0.1 M NaH$_2$PO$_4$ contains 1 mol of pyridoxal-5-P per dimer and its enzymatic activity is increased by addition of pyridoxal-5-P as shown in Fig. 3C.

A change in the concentration of NaH$_2$PO$_4$ from 0.1 M to 1 M results in the elution of a third peak devoid of enzymatic activity. After addition of pyridoxal-5-P, this protein recovers full enzymatic activity. Since the elution profile of this protein coincides with that of a sample of apotransaminase run under similar experimental conditions, it seems reasonable to propose that it is apoprotein (Fig. 3B).

Judging from the relative distribution of the protein species in the elution pattern, it appears that the protein species containing 1 mol of PLP per dimer represent approximately 60% of the total protein eluted from the column; while the protein species, apotransaminase-2PLP and apotransaminase, represent 40% of the total protein content. This type of protein distribution pattern can not be explained in terms of cooperative interaction between the binding sites. Indeed, positive interaction between the pyridoxal-5-P binding sites would result in the formation of larger amounts of reconstituted aminotransferase (apotransaminase-2PLP), while the actual elution profile does not contain any increase in enzymatic activity upon addition of pyridoxal-5-P. The elution profile of this protein coincides with that of a sample of apotransaminase run under similar conditions, it seems reasonable to propose that it is apoprotein (Fig. 3B).

Further support for the hypothesis that the binding of pyridoxal-5-P is not highly cooperative was derived from affinity chromatography experiments conducted on samples of apoenzyme preincubated with 1 mol of pyridoxal-5-P per dimer and then subjected to reduction with NaBH$_4$. Fig. 3D shows the results obtained with the reduced sample after affinity chromatography on Sepharose derivatized with N'-(o-aminohexyl)-PMP. The fraction eluted at pH 8 does not show any increase in enzymatic activity upon addition of pyridoxal-5-P. The elution profile of this protein coincides with that of the holoenzyme. The protein eluted with 0.1 M NaH$_2$PO$_4$ contains 1 mol of P-pyridoxal per dimer and recovers 50% of the aminotransferase activity after addition of pyridoxal-5-P. This protein represents approximately 70% of the total protein eluted from the column. The fraction eluted with 1 M NaH$_2$PO$_4$ is enzymatically inactive, but recovers transaminase activity after addition of pyridoxal-5-P. Its elution profile coincides with that of the apoenzyme which is eluted from the Sepharose column by 1 M NaH$_2$PO$_4$ (Fig. 3B). It is interesting to note that the protein distribution pattern of the reduced enzyme strongly resembles the distribution pattern of the apotransaminase reconstituted with 1 mol of pyridoxal-5-P.

**DISCUSSION**

The subject of cooperativity in ligand binding to specific sites in oligomeric enzymes has been extensively discussed in recent years. Models to explain the phenomena of either positive or negative cooperativity have been proposed by several authors (13–15). More recently, an extreme example of negative cooperativity, i.e. half-site reactivity has been detected in several enzymes (16, 17). In such cases, the maximal stoichiometry yield of either an enzyme substrate-covalent intermediate or of a product in a single turnover amounts to only one-half the number of apparently equivalent active sites. An interesting example of half-site reactivity constitutes the enzyme horse alcohol dehydrogenase investigated by Bernhard and co-workers (16).

In spite of the extensive research work done with the dehydrogenases, few studies designed to test the hypothesis of cooperative interaction between the binding sites of P-pyridoxal enzymes have been reported in the literature. The aim of the experiments described in this paper is to investigate to what extent the interaction between the protomers of mitochondrial aspartate aminotransferase influences the affinity of the binding sites of the protein for either the inhibitor 4-pyridoxic-5-P or the cofactor pyridoxal-5-P. The data obtained from the fluorometric titrations support the concept that the enzyme aspartate aminotransferase contains a set of independent binding sites. Furthermore, the interaction of the cofactor with one-half of the catalytic sites available does not influence the affinity of the remaining binding sites for the inhibitor 4-pyridoxic-5-P.

However, some caution must be exercised in the interpretation of the fluorometric titrations since it is difficult to detect cooperative effects when the difference of ligand affinities among sites is relatively small.

In addition, a mixture of several isoenzymes with different ligand affinities can give an apparent negative cooperativity even in the absence of protomer interactions.

In order to avoid uncertainties in the interpretation of titration data, we decided to resort to a method that permits the separation and identification of the enzyme species formed when one half of the catalytic sites of the aminotransferase are saturated with pyridoxal-5-P.

It is shown in the present work that affinity chromatography on Sepharose derivatized with N'-(o-aminohexyl)-PMP is the method suitable for this type of research. Indeed, a careful analysis of the elution pattern permits a quantitative determination of the relative distribution of enzyme species present in the system. Thus, the data obtained from affinity chromatography experiments indicate that the relative distribution of the enzyme species, apoprotein-2PLP, apoprotein-1PLP, and apoprotein (2:6:2) does not differ significantly from the binomial distribution (2.5:5:2.5) expected for an enzyme that contains two independent binding sites. On the basis of these results, it seems reasonable to conclude that protomer interaction in aspartate aminotransferase does not bring about either positive or extreme negative cooperativity between the binding sites.

**REFERENCES**

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