THE APO/HOLO HYBRID OF CYTOSOLIC ASPARTATE AMINOTRANSFERASE, PREPARATION AND STUDIES ON SUBUNIT INTERACTIONS

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SUMMARY. The apo/holo hybrid dimer of cytosolic aspartate aminotransferase from pig heart has been isolated by isoelectric focusing from a semi-reconstituted apo enzyme preparation. Comparison of the catalytic center activity and of the optical features of the coenzyme chromophore of the hybrid containing one active center with that of the homomer containing two revealed no differences in these properties and suggests that the subunits function independently from one another. However, differential thermal inactivation studies of hybrids and of homomers showed that the subunit carrying the coenzyme markedly stabilizes the structure of the neighboring subunit. Thus, coenzyme binding elicits functionally mute conformational changes which extend to and across the subunit interface.

Cytosolic aspartate aminotransferase is a dimeric protein with identical subunits. Each subunit is composed of 412 amino acid residues (1) and carries as coenzyme a molecule pyridoxal 5'-phosphate. The transamination follows a double displacement mechanism in the course of which the enzyme shuttles between the pyridoxal and the pyridoxamine form (2,3). Distinct differences in the protein conformation of the pyridoxal and the pyridoxamine form have been established by various experimental approaches (4, 5,6). Syncatalytic changes in the susceptibility toward chemical modification of cysteinyl residue 390 indicate that the conformational transition is a multistep process accompanying catalysis (5,7).

In the present study the effect of the coenzyme on subunit conformation and subunit interactions was explored. The apo/holo hybrid dimer (one apo subunit and one subunit carrying the coenzyme) was isolated by isoelectric focusing from a semi-reconstituted apo enzyme preparation. The structural and functional properties of the apo/holo hybrid on the one hand and of the apo and holo homomers on the other were compared and evaluated with respect to manifestations of subunit interactions.

MATERIALS AND METHODS. The α-subform of cytosolic aspartate aminotransferase (EC 2.6.1.1) was isolated from pig heart according to the procedure of Banks et al. (8) as modified in our laboratory (9). Pyridoxal 5'-phosphoric acid and pyridoxamine 5'-phosphate hydrochloride were obtained from Merck, L-cysteine sulfinic acid from Sigma. Protein concentration was determined spectrophotometrically using a molar absorptivity of the dimer $\varepsilon_{280} = 1.4 \times 10^5\ M^{-1}\ cm^{-1}$. The specific activity of the enzyme was 360 U/mg, when assayed in the coupled assay with malate dehydrogenase (EC 1.1.1.37, obtained from Boehringer) as described previously (5). The pyridoxamine form of the enzyme was prepared by addition of 20 mM cysteine sulfinic acid (10) and subsequent gel filtration on a column of Sephadex G-25 equilibrated with 50 mM sodium phosphate, pH 7.5. For the preparation of the apo enzyme, the pyridoxamine form of the enzyme was dialyzed twice for 15 hours against 500 volumes 0.5 M sodium phosphate, pH 5.1. The specific activity of the resulting apo enzyme preparation was < 0.5 per cent of the initial value.

Absorption and circular dichroism spectra were recorded at room temperature with a Cary 15 spectrophotometer and a Cary 61 spectropolarimeter, respectively. pH-Values were determined at room temperature with a pH meter 26 from Radiometer.

Isoelectric focusing was performed with a 110 ml column from LKB at 4° with 1.2 per cent Ampholine (LKB) of pH 5.3 - 6.1, which had been preisolated from the commercially available pH 5 - 7 Ampholine (11). For filling of the focusing column a linear sucrose gradient containing the ampholytes (mixing vessel 45 ml of 0.6 per cent ampholytes in water, reservoir vessel 45 ml of 1.8 per cent ampholytes in 40 per cent sucrose) was pumped onto the anode solution (1 per cent sulfuric acid in 50 per cent sucrose). The samples, not exceeding 10 ml and 30 mg of protein in 20 per cent sucrose were introduced into the middle of the gradient. The cathode solution (2 per cent triethanolamine) was finally layered onto the top of the gradient. Using maxima of 600 V and 2.5 mA, the protein bands reached stable positions in the pH gradient after 15 to 18 days and were subsequently eluted. With 1200 V the same results were obtained in 10 to 12 days. For elution 1-ml fractions were collected. The frac-

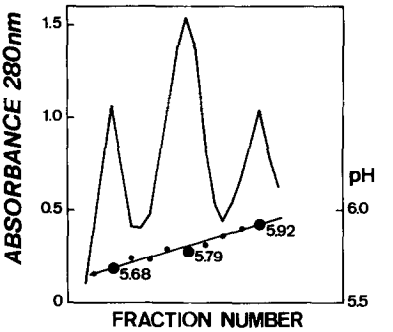


Fig. 1 Isoelectric focusing of an apo enzyme preparation semi-reconstituted with pyridoxal 5'-phosphate. The conditions for semi-reconstitution are described in the text, the procedure used for isoelectric focusing is given in the Methods section. The yield both for protein and enzymatic activity after focusing was > 90 per cent.

tions of the separate peaks were pooled and protein was separated from the ampholytes on a 2 x 40 cm Sephadex G-25 fine column, equilibrated with 50 mM sodium phosphate, pH 7.5.

RESULTS. For semi-reconstitution, pyridoxal 5'-phosphate (0.2 µmol dimer) was added to the apo enzyme (0.2 µmol dimer) in 6 ml 50 mM sodium phosphate, pH 7.5, at room temperature. Within 90 min the specific activity increased to a constant value of 180 U/mg, i.e. 50 per cent of that of the original holo enzyme. Separation and quantitative determination of the different dimer species in this reaction mixture were obtained by isoelectric focusing in a shallow pH gradient (Fig. 1). The elution diagram shows three peaks positioned at pH 5.68, 5.79 and 5.92. The peaks were identified by means of their position in the pH gradient, their specific activity and their absorption and circular dichroism spectra. The peak at pH 5.68 exhibits a specific activity of 360 U/mg and its position in the pH gradient corresponds to that of individually focused pyridoxal homomer; on the other hand the peak at pH 5.92 corresponds to the position of individually focused apo homomer and has a specific activity of 35 U/mg which on addition of pyridoxal 5'-phosphate is increased to 360 U/mg. The specific activity of the center peak, representing the apo/pyridoxal hybrid, is 180 U/mg, addition of pyridoxal 5'-phosphate increases its activity to 360 U/mg. The distribution ratio of protein in

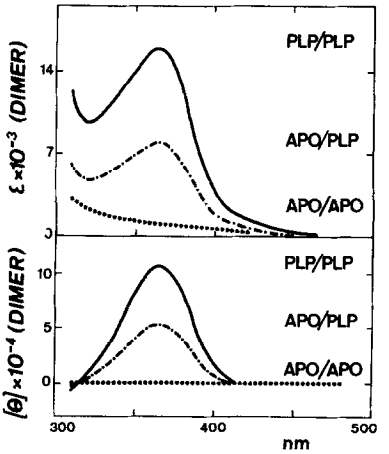
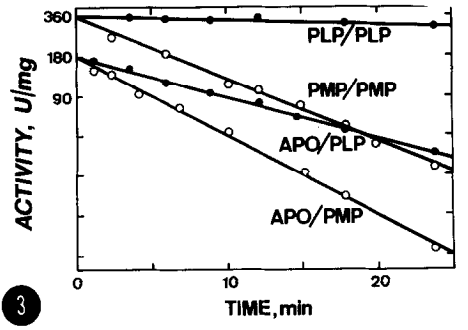


Fig. 2 Absorption and circular dichroism spectra of pyridoxal (PLP/PLP) and apo (APO/APO) homomer and of apo/pyridoxal (APO/PLP) hybrid. Absorption spectra were obtained with 4 µM enzyme (dimer concentration) in 50 mM sodium phosphate, pH 7.5, at room temperature in 4-cm cuvettes, and the circular dichroism spectra at the same conditions in 5-cm cuvettes.

the three peaks is approximately 3:5:3. Refocusing of the hybrid yields one main peak flanked by two small ones, each containing about 5 per cent of the total protein. The main peak again contains the hybrid and the two adjoining peaks contain the corresponding homomers.

Both the absorption and the circular dichroism spectrum of the apo/pyridoxal hybrid correspond with the arithmetical mean of the spectra of the apo and of the pyridoxal homomer (Fig. 2). With an excess of cysteine sulfinate (see Methods) the apo/pyridoxal hybrid is converted completely to the apo/pyridoxamine hybrid. Again, the absorption and the circular dichroism spectrum of the coenzyme chromophore of this hybrid correspond with the arithmetical mean of the corresponding homomers (not shown). Thus, the holo subunit of the hybrid dimer is indistinguishable from a holo subunit in the holo homomers with regard to both the catalytic center activity and optical properties of the coenzyme chromophore.

On the other hand, differential thermal inactivation shows marked coenzyme-dependent subunit interactions. The rate of heat



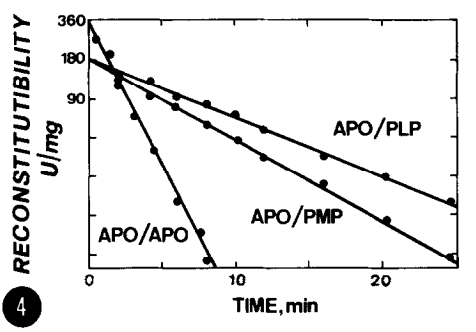


Fig. 3 Rate of thermal inactivation of the holo subunits in pyridoxal (PLP/PLP) and pyridoxamine (PMP/PMP) homomers, and in apo/pyridoxal (APO/PLP) and apo/pyridoxamine (APO/PMP) hybrids. Enzyme solutions (0.5 ml, 1.1 µM dimer concentration in 50 mM sodium phosphate, pH 7.5, equilibrated at room temperature) were incubated in a 70° waterbath. Time-measuring was started after one minute of incubation, when the temperature had reached ∿ 68°. At the indicated times 10 µl aliquots were assayed directly for aminotransferase activity at 25° (5). The progress curves of the assays were linear in all experiments. The presence of a 20-fold molar excess of free coenzyme during heat treatment decreased the rate of inactivation of both the pyridoxal and the pyridoxamine homomer approximately twofold.

Fig. 4 Rate of thermal denaturation of the apo subunits in the apo/pyridoxal (APO/PLP) and apo/pyridoxamine (APO/PMP) hybrids and in the apo homomer (APO/APO). The reconstitutibility of the heat-treated enzymes, i.e. the difference in activity before and after incubation with pyridoxal 5'-phosphate is plotted as a function of the duration of the heat treatment. The conditions for heat treatment were the same as those described in the legend of Fig. 3. At the indicated times, two aliquots of 10 µl were analyzed; one was assayed directly for aminotransferase activity, the other one was added to 50 µl 0.1 mM pyridoxal 5'-phosphate and after 90 minutes at room temperature assayed for enzymatic activity. Prolonged incubation with pyridoxal 5'-phosphate did not increase the activity any further.

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inactivation of the pyridoxal homomer is 9 times slower than that of the pyridoxal subunit in the apo/pyridoxal hybrid (Fig. 3). The mutual stabilization of holo subunits is less pronounced in the case of pyridoxamine subunits, the pyridoxamine homomer being only slightly more stable than the apo/pyridoxamine hybrid. The pyridoxal homomer is 14 times more stable against heat denaturation than the pyridoxamine homomer. The heat denaturation of

the apo subunit was followed by determining the time-dependent loss of its reconstitutibility with pyridoxal 5'-phosphate (Fig. 4). The rate of denaturation of the apo subunit in the apo/pyridoxal hybrid is 4 times, in the apo/pyridoxamine hybrid 3 times slower than the rate of denaturation of the apo homomer. Apparently, in the apo/holo hybrid the apo subunit is stabilized by the neighboring holo subunit.

DISCUSSION. The preparation of homogenous apo/holo (pyridoxal or pyridoxamine) hybrid dimers of cytosolic aspartate aminotransferase provides a novel approach to the study of coenzyme-dependent subunit interactions in this enzyme. The mass ratio of pyridoxal homomer, apo/pyridoxal hybrid, and apo homomer in a semi-reconstituted apo enzyme preparation approximates a binominal distribution and thus indicates independent binding of pyridoxal 5'-phosphate to the two active sites of the enzyme dimer. The equivalence of apo/holo hybrid and holo homomer with respect to optical properties of the coenzyme chromophore and, importantly, to catalytic center activity further demonstrates the mutual independence of the two active sites. Quite analogous results have been obtained with the holo/reduced hybrid of the enzyme (one holo subunit and one subunit with the internal aldimine reduced by $NaBH_4$) (9). These data conclusively rule out any mechanism of transamination as catalyzed by this enzyme which would involve interactions between the two active sites as an integral feature. Using a similar experimental procedure the same conclusion has been reached by Lee and Churchich for the mitochondrial aspartate aminotransferase from beef liver (12).

The thermal inactivation experiments confirm the difference in conformation of the enzyme in its different functional states (cf. 4-6). The stabilization of both holo or apo subunits by a neighboring holo subunit shows that the coenzyme-induced conformational changes extend to the subunit interface and involve parts of the adjoining subunit. Participation of the subunit interface in coenzyme-induced conformational alterations of the enzyme protein was also found in a study on the stability of the quaternary structure of the enzyme. Whereas subunit interchange between pyridoxal and the pyridoxamine homomers is quite rapid

(equilibrium being reached within < 5 min), the apo enzyme and the enzyme with $NaBH_4$ reduced internal aldimine show virtually no subunit interchange with pyridoxal homomer within days (9). Coenzyme-dependent changes in the quaternary structure have also been observed in other pyridoxal dependent enzymes (13 - 17).

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