Aspartate aminotransferase (α subform from pig heart cytosol), a dimeric enzyme composed of two identical subunits, was tested for subunit interactions imposed on the transamination reaction.

The two functional variants of the enzyme arising from its double displacement mechanism, i.e. the pyridoxal-5'-P form (LL dimer) and the pyridoxamine-5'-P form (MM dimer), as well as the enzyme whose aldime bonds to pyridoxal-5'-P had been reduced with sodium borohydride (RR dimer), were found to be separable by isoelectric focusing. Separation of a half-reduced pyridoxal-5'-P enzyme preparation yielded a 1:2:1 distribution of LL homomer, LR hybrid, and RR homomer (isoelectric points 5.68, 5.97, and 6.03, respectively). The enzymatic activity of the isolated LR hybrid is exactly half that of the native LL homomer.

Labeling of the L subunits by reduction with sodium borohydride was employed to determine the distribution of L and M subunits among aspartate aminotransferase dimers at transamination equilibrium. The substrate pair 2-ketoglutаратate and glutamate in a concentration ratio appropriate to result in the formation of equimolar concentrations of L and M subunits was added to a solution of the enzyme. After removal of the substrates to substoichiometric concentrations, the LL/LM/MM dimer mixture was reduced with sodium borohydride to a RR/RL/LL mixture, which was converted to a readily separable RR/RL/LL mixture by the addition of excess 2-ketoglutaratate. Isoelectric focusing yielded a 1:2:1 distribution of LL homomer (originally MM), LR hybrid (originally ML), and RR homomer (originally LL). The binomial active site occupancy pattern demonstrates random interconversion of the individual L and M subunits of the aspartate aminotransferase dimers during transamination, i.e. independent catalytic function of the two active sites of the aspartate aminotransferase dimer.
RESULTS

The experimental objective of this investigation was to measure the relative concentrations of LL homomer, LM hybrid, and MM homomer in a population of aspartate aminotransferase dimers after attainment of the transamination equilibrium. In an enzyme solution that, after removal of the substrates, contains equal concentrations of L and M subunits, a 1:2:1 distribution of the three dimer species would be expected if the two subunits of the enzyme underwent transamination independently from each other. Any deviation from the binomial distribution would indicate the existence of functionally important subunit interactions.

Three variants of the enzyme were employed in the present study, the LL, the MM, and the enzymically inactive RR dimer prepared from the LL homomer by reduction with sodium borohydride. On isoelectric focusing the three homomers, analyzed separately or combined, reproducibly accumulate in distinct bands at defined positions in the pH gradient, the LL homomer at pH 5.68 and the RR and MM homomers in close proximity at pH 6.03 and 6.07, respectively (S.D., ±0.05 pH unit). These pH values correspond approximately to the isocionic pH values of the enzyme dimers, as indicated by their proton titration curves (see Fig. 1S in miniprint supplement).

The LR hybrid could readily be isolated from a LL homomer preparation that had been half-reduced with sodium borohydride. Isoelectric focusing separated the half-reduced dimer mixture into three components with a distribution of protein of 1:2:1 (Fig. 1). The position in the pH gradient, the absorption spectrum, and the specific activity showed Peak I to be the LL homomer (350 units/mg) and Peak III to be the RR homomer (26 units/mg). The center peak II was identified as the LR hybrid; its pyridoxal-5'-P content was 1.0 mol/mol of dimer, its absorption spectrum (in 50 mM sodium phosphate (pH 7.5)) corresponded exactly to the arithmetical mean of those of the LL and RR homomers, and its specific activity was 180 units/mg (i.e. half of that of the LL homomer). On addition of glutamate (10 mM) or cysteine sulinate (2 mM), the LR hybrid was converted to the MM hybrid, whose absorption spectrum again was indistinguishable from that of an equimolar mixture of MM and RR homomer. Refocusing of the isolated LR hybrid after storage for 6 days at 4°C in 50 mM sodium phosphate (pH 7.5), yielded one symmetrical peak at the same position in the pH gradient with unchanged specific activity and spectral properties. Similarly, isoelectric focusing of an equimolar mixture of LL and RR homomers (28 µm dimer each in 50 mM sodium phosphate (pH 7.5)) that had been kept for 10 days at room temperature yielded unaltered LL and RR homomers without hybrid having been formed. Analogous experiments showed that the MR hybrid and the MM and RR homomers also do not interchange their subunits.

Attempts to isolate the LM hybrid by a procedure analogous to that used for the LR hybrid were unsuccessful. The substrate pair 2-ketoglutarate plus glutamate was added to an enzyme solution in saturating concentrations at such a ratio that the enzyme, after removal of the substrates by gel filtration, contained equimolar concentrations of L and M subunits. Isoelectric focusing of this transamination equilibrium mixture yielded LL and MM homomers, each representing approximately half of the total protein. A small intermediary peak proved to be subject to a time-dependent decrease during the focusing run (see Fig. 2 in miniprint supplement). Its absorption spectrum coincided with that of an equimolar mixture of the two main peaks, and its pyridoxal-5'-P content was 1.0 mol/mol of dimer. More rapid separation of the transamination equilibrium mixture by isoelectric focusing on thin layer polyacrylamide gel that requires only 90 min for completion yielded three about equally strong bands (see Fig. 3 in miniprint supplement). Apparently, the center peak contains LM hybrid that, during isoelectric focusing, is continuously transaminated into LL and MM homomers either by mutual transamination of L and M subunits mediated by contaminating traces of substrates or by interchange of holo subunits, or both (see miniprint supplement). The stability of the LR hybrid, however, allowed determination of the relative concentration of the LM hybrid in a transamination equilibrium mixture by an indirect procedure (Scheme 1).

![Figure 1. Isoelectric focusing of a half-reduced LL homomer preparation. Half of the internal aldmines of LL enzyme (0.18 µmol of dimer) were reduced with sodium borohydride prior to isoelectric focusing. The degree of reduction was gauged by the decrease in enzymatic activity and the spectral changes (13). Peaks I to III reached constant positions in the pH gradient (---) at pH values of 5.68 (LL homomer), 5.86 (LR hybrid), and 6.03 (RR homomer), respectively. The absorbance at 280 nm (-----) was corrected for the absorbance of the ampholytes. The pooled fractions of each peak were freed from ampholytes and characterized with respect to protein content, pyridoxal-5'-P content, enzymic activity, and absorption spectrum.](http://www.jbc.org/)

**SCHEME 1**

The MM/LM/LI. transamination equilibrium mixture containing equal concentrations of L and M subunits was treated with excess sodium borohydride to reduce the coenzyme-enzyme aldimine linkages. Prior to reduction, the substrate pair was removed by gel filtration. Otherwise, all enzyme dimers would finally have been converted into RR dimers via transamination of the M subunits with 2-ketoglutarate and reductive trapping of the ensuing L subunits. As a consequence of the reduction, the specific enzymic activity of the dimer mixture decreased to half of its initial value. Since isoelectric
The present approach to the detection of functionally important subunit interactions in oligomeric enzymes is based on the determination of the active site occupancy pattern in a catalytically engaged enzyme population. In principle, the approach is feasible if two requirements are met: firstly, there must be means to label the catalytically engaged subunits, e.g. by stabilization of covalent enzyme substrate intermediates or by the use of substrate analogs that react irreversibly with the enzyme. Secondly, the labeled enzyme must be physically separable from the unoccupied enzyme. Aspartate aminotransferase, in view of its double displacement mechanism, would prima facie appear to be directly amenable to the determination of the active site occupancy pattern; its two functional states, the L and the M forms, should be stabilizable by the mere removal of the transaminating substrates. However, the slow disappearance of the LM hybrid during isoelectric focusing necessitated stabilization of the transamination equilibrium mixture prior to isoelectric focusing by reduction with sodium borohydride.

Two types of functionally important subunit-dependent subunit interactions may occur in enzymes, viz. positive or negative cooperative effects in substrate binding and, more speculatively, interactions operative during the covalency changes resulting in synchronized or reciprocating action of the subunits. Either type of subunit interaction would manifest itself by a deviation of the active site occupancy pattern from a binomial distribution. The detectability of subunit interactions by the present approach depends on the concentration of the subunits present at the moment of reduction with sodium borohydride. At saturating concentrations, when the covalent transformation of the substrate is rate limiting, subunit interactions operative in this segment of the enzymatic reaction might preferably become manifest; low substrate concentrations, on the other hand, would favor the detection of interactions in binding of the substrate. In the present experiments, two procedures for the preparation of the transamination equilibrium mixture were employed: (a) addition of glutamate and 2-ketoglutarate in saturating concentrations and subsequent gel filtration, and (b) addition of the substrates in low substoichiometric concentrations to an equimolar mixture of LL and MM homomers. In both procedures, the reduction of the transamination equilibrium mixture occurs in the presence of low, subsaturating concentrations of the substrates. According to the above-mentioned considerations, the observed binomial active site occupancy pattern excludes the existence of subunit interactions on the binding level, whereas the existence of weak interactions involving the covalent phase of catalysis is not completely ruled out.

The structural and functional properties of the isolated LR hybrid are fully compatible with functional independence of the two subunits of aspartate aminotransferase with respect to both the binding and the covalent transformation of the substrates. Cytosolic aspartate aminotransferase from pig heart has been reported to retain its dimeric structure also in very dilute solution (1 × 10^{-10} M) (16). Thus, the LR hybrid may be assumed to have remained a dimer at the concentrations used in this study to measure enzymic activity or to record the absorption spectra. In both the LR and MR hybrids, the absorption spectra of the coenzyme chromophores serving as intrinsic optical probes of the active site region correspond with the arithmetic mean of those of the two respective homomers. Further, the unchanged catalytic activity of the nonreduced subunit, as well as the random occurrence of the reduction, testify to the absence of functionally significant subunit interactions. Similar observations have been made in previous experiments with an LR hybrid obtained by hybridization of LL and chemically modified RR homomers under denaturing conditions (9, 10).

In conclusion, two different experimental approaches have demonstrated the mutual functional independence of the two active sites of cytosolic aspartate aminotransferase from pig heart: (a) studies with isolated hybrid dimers containing one active and one inactive subunit (apo (see the introduction) or with reduced internal aldimine); and (b) determination of the active site occupancy pattern in the fully active enzyme dimer at transamination equilibrium.

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