

Syncatalytic conformational changes in aspartate aminotransferase determined by hydrogen-deuterium exchange

(mechanism of enzyme action/transamination/pyridoxal 5'-phosphate/enzyme flexibility/free energy transduction in enzymes)

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ABSTRACT Catalysis-linked conformational transitions of aspartate aminotransferase (cytosolic isoenzyme from pig heart; L-aspartate:2-oxoglutarate aminotransferase, EC 2.6.1.1) have been probed by infrared spectrophotometric measurement of hydrogen-deuterium exchange. In the unliganded pyridoxal form of the enzyme at pH 6.0 and 20°, 43% of the total 411 peptide hydrogens per subunit exchange within the first 10 min. An additional 9% exchange slowly in the following time period to 360 min. A quite similar exchange curve is obtained with the pyridoxamine form of the enzyme, indicating close correspondence in conformation of both unliganded forms of the enzyme. Formation of a nonproductive adsorption complex of the pyridoxal enzyme with 2-oxoglutarate or of the pyridoxamine enzyme with glutamate alters the exchange characteristics only slightly. In contrast, the formation of an equilibrium mixture of the covalent transamination intermediates, which occurs in the simultaneous presence of the amino acid and the keto acid substrate, results in a marked retardation of hydrogen exchange, reflecting a substantial tightening of the structure of the enzyme. The exchange reactions of at least 26 peptide hydrogens per subunit (6% of the total) are retarded by a factor of 6 on the average. The occurrence of such syncatalytic conformational changes reflects energetic coupling of the covalency changes at the active site with conformational changes of the macromolecular protein matrix that may contribute to optimizing the free energy profile of enzymic transamination.

Conformational changes of enzymes concomitant to the binding of substrate or other specific ligands are amply documented by experiment. The changes are established as the basis for mechanisms of regulation and are also thought to contribute to both the specificity and efficiency of enzymic catalysis (1, 2). The occurrence of conformational adaptations of enzymes during the subsequent phase of catalysis, i.e., during the covalency changes, has been postulated on the basis of thermodynamic arguments (3, 4). Syncatalytic adjustment of the conformation of an enzyme might optimize the free energy profile of the bond rearrangement steps. However, experimental evidence for such transient changes in conformation is scarce; their detection might be possible only under especially favorable circumstances.

In aspartate aminotransferase (L-aspartate:2-oxoglutarate aminotransferase, EC 2.6.1.1) the occurrence of syncatalytic conformational changes has been indicated by differential chemical modifications. Both in the mitochondrial isoenzyme from chicken and pig (5) and in the cytosolic isoenzyme from pig (6), the reactivity of a nonessential cysteinyl residue toward sulfhydryl reagents is enhanced by one or two orders of magnitude, respectively, when the enzyme undergoes the bond rearrangement steps of transamination. In the present study, the conformational behavior of aspartate aminotransferase

during catalysis was explored by hydrogen-deuterium exchange, a method gauging the reactivity of the amide hydrogens of all peptide bonds of the protein and thus uniquely suited for detecting subtle changes in the conformation of the protein matrix (see refs. 7, 8).

EXPERIMENTAL PROCEDURE

Aspartate Aminotransferase. The α subform of cytosolic aspartate aminotransferase was isolated from pig heart according to the procedure of Banks *et al.* (9) as modified in our laboratory. Protein concentration was determined photometrically using a molar absorptivity of the dimer $\epsilon_{280} = 1.4 \times 10^5 \text{ M}^{-1} \text{ cm}^{-1}$. The specific activity of the enzyme was 360 units/mg as determined in the coupled assay with malate dehydrogenase (6). It was decreased to 45% of this value when D_2O was substituted for water in the assay. The pyridoxamine form of the enzyme was prepared from the pyridoxal form by addition of 4 mM cysteine sulfinate (10) and subsequent gel filtration.

Chemicals. L-Glutamic acid, L-aspartic acid, and 2-oxoglutaric acid were obtained from Fluka; 2-methyl-DL-aspartic acid and L-cysteine sulfinic acid were from Sigma; pyridoxal 5'-phosphoric acid and pyridoxamine 5'-phosphoric acid hydrochloride were from Merck; DL-erythro-3-hydroxyaspartic acid was from Calbiochem; D_2O (99.75%) was from the Centre d'Etudes Nucléaires de Saclay; malate dehydrogenase (EC 1.1.1.37) and NADH were from Boehringer.

Measurement of Hydrogen-Deuterium Exchange. The exchange of peptide hydrogens with deuterium was followed by infrared spectrophotometry (7, 11). Samples of an enzyme solution (800-1500 μl , 18 mg/ml in 50 mM sodium phosphate) were adjusted to the desired pH values and lyophilized in microvessels. At zero time the lyophilized protein was dissolved in the original volume of D_2O (800-1500 μl) and kept at $20 \pm 0.1^\circ$. The activity of the redissolved enzyme was the same as before lyophilization. All indicated pH values refer to the values measured in the aqueous solutions before lyophilization and dissolution in D_2O . The decrease in absorption of the NH (amide II) band at 1545 cm^{-1} was followed as a function of time. The principal absorption band of the peptide group at 1650 cm^{-1} (amide I) is mainly due to the C=O stretching motion, and its amplitude remains constant on deuteration. Thus, the absorbance ratio amide II/amide I may serve as a measure of the number of unexchanged peptide hydrogens (11). For spectrophotometry a Beckman infrared spectrophotometer model 20A was used. Samples of about 100 μl were measured in a calcium fluoride cell (0.1-mm path length). The reference cell was filled with D_2O or D_2O plus substrates (see description of individual experiments). The amide II band was scanned from 1560 to 1530 cm^{-1} (scan speed $6 \text{ cm}^{-1}/\text{min}$). The indi-

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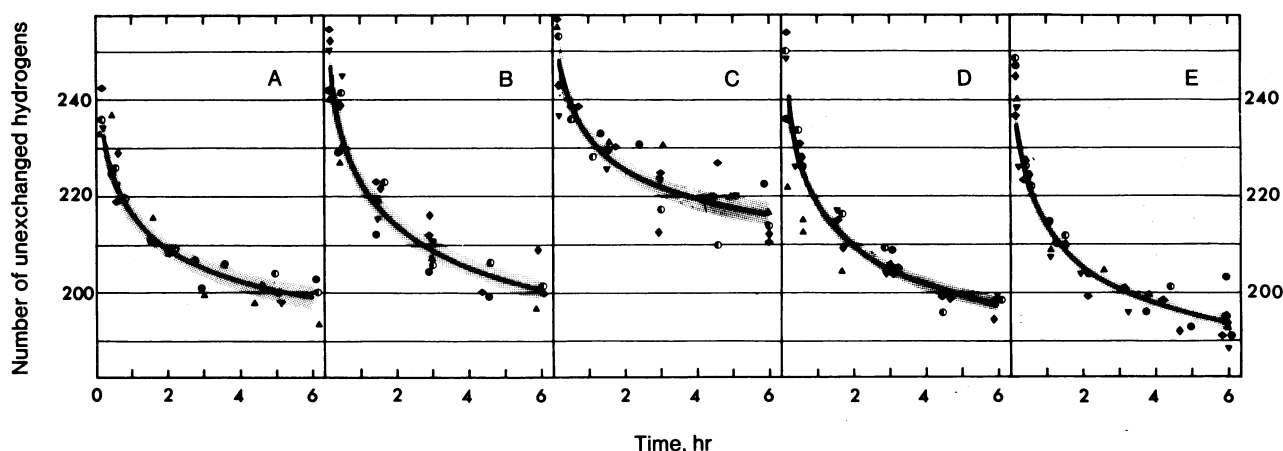


FIG. 1. Hydrogen-deuterium exchange curves of aspartate aminotransferase in different functional states: (A) unliganded pyridoxal form, (B) its nonproductive adsorption complex, (C) the covalent intermediates, (D) the nonproductive adsorption complex of the pyridoxamine form, and (E) the unliganded pyridoxamine form. The experiments were carried out at 20° in 50 mM sodium phosphate, pH 6.0. In the case of the unliganded forms (A and E) the exchange reactions were started by dissolving the lyophilized enzyme in D₂O and in the other cases by dissolving it in D₂O containing either 2 mM 2-oxoglutarate (B), 2 mM 2-oxoglutarate plus 70 mM glutamate (C), or 70 mM glutamate (D). Beforehand, the substrates had been dissolved in H₂O, adjusted to pH 6.0, lyophilized, again dissolved in D₂O, and lyophilized for a second time. Their solutions in D₂O served as reference solutions. In the diagrams each set of a symbol refers to an individual exchange experiment. The center curve represents the statistically ascertained exchange curve (see *Experimental Procedure*) and the shaded area indicates the standard error of the mean.

cated times refer to the moment at which the maximum of the amide II band was passed. Next, the amide I band at 1650 cm⁻¹ was scanned from 1660 cm⁻¹ to 1640 cm⁻¹.

Calculation of the Number of Unexchanged Hydrogens. The amide II/amide I absorbance ratio of undeuterated aspartate aminotransferase was 0.68 as deduced from spectra of the lyophilized protein in potassium bromide pellets. The amide II/amide I absorbance ratio of the fully deuterated protein was 0.12 as determined after incubation for 6 hr at 70° in 1% sodium dodecyl sulfate/50 mM sodium phosphate in D₂O at pH 6.0 (background ratio). Thus, the number of unexchanged hydrogens out of a total of 411 peptide hydrogens per subunit (12, 13) was obtained from the relationship $n = 411 (A_{\text{amide II}}/A_{\text{amide I}} - 0.12)/(0.68 - 0.12)$ and plotted as a function of time.

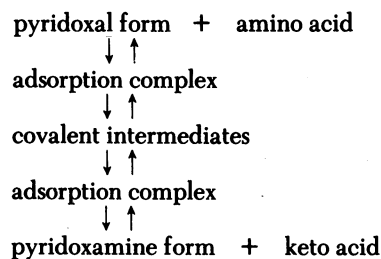
For each functional form of the enzyme, exchange data were obtained in three separate experiments, each determined in duplicate. The exchange curves were ascertained by linear regression of the logarithmically transformed values of the quotient $A_{\text{amide II}}/A_{\text{amide I}}$ and the duration of exchange (for details see ref. 14). The transformed exchange curves of the different functional forms of the enzyme were tested for significance of their differences in slope and vertical distance. A significant difference ($P \leq 0.05$) in the exchange curves was found between the unliganded pyridoxal and pyridoxamine enzyme, and between both unliganded enzyme forms and the corresponding nonproductive adsorption complexes as well as the covalent intermediates with substrates.

Interpretation of the Exchange Curves of Aspartate Aminotransferase. Each experimental curve represents the sum of the first-order decay functions of the individual peptide hydrogens, which in aqueous solution are believed to undergo continuous conformational fluctuations between nonexchanging and exchanging states (7, 8). On the basis of studies at different pH values (15), the hydrogen-deuterium exchange of aspartate aminotransferase (14) was found to follow the high-motility or EX-2 mechanism of Linderstrøm-Lang (7). In this model intramolecular conformational fluctuations are rapid compared to the pseudo-first-order exchange reaction of the exposed peptide hydrogens with the solvent. As a con-

sequence the rates of exchange observed are dependent upon the extent of exposure of each peptide hydrogen, i.e., upon the conformational equilibrium determining it. Thus, changes in velocity of exchange upon modulation of the functional state of the protein can be interpreted as being due to changes in conformational equilibria, the difference between the exchange curves relating to the extent of their displacement.

RESULTS

The hydrogen-deuterium exchange characteristics of aspartate aminotransferase were determined in its different functional states, i.e., the two unliganded enzyme forms, the enzyme-substrate adsorption complexes, and the covalent enzyme-substrate intermediates. Enzymic transamination follows a double-displacement mechanism:



In the presence of a pair of structurally analogous substrates, such as glutamate and 2-oxoglutarate, transamination occurs without any change in the concentration of either substrate and all functional states of the enzyme are present in equilibrium concentration. Each substrate was used at a concentration 5 times its K_m value (16), thus ensuring the formation of a balanced transamination equilibrium mixture containing equal concentrations of the two unliganded forms and of the adsorption complexes. The productive enzyme-substrate adsorption complexes could not be examined for obvious reasons; they were substituted by the nonproductive enzyme-substrate complexes. As is apparent from the exchange curves of the different functional states of the enzyme (Fig. 1), observation

with infrared spectroscopy under the present conditions (pH 6.0, 20°) focuses on the exchange behavior of ~13% of the total 411 peptide hydrogens per subunit. In all functional states close to 40% of the peptide hydrogens exchange too rapidly to be followed and 47% have not exchanged after 6 hr, when the experiments were terminated.

The exchange curve of the unliganded pyridoxal form (Fig. 1A) is similar in shape to that reported in a previous study in which the hydrogen-deuterium exchange characteristics of holo and apo aspartate aminotransferase were compared (17). The high proportion of about 47% peptide hydrogens remaining unexchanged after 6 hr is indicative of a relatively compact protein structure (cf. ref. 11). The unliganded pyridoxal form and the unliganded pyridoxamine form exchange exactly the same number of hydrogens within the first 30 min (Fig. 1 A and E). However, the forms diverge somewhat with respect to the more slowly exchanging hydrogens; 200 peptide hydrogens have exchanged in the pyridoxamine form at about 3 hr and in the pyridoxal form at about 5 hr. Formation of the nonproductive adsorption complexes of both enzyme forms slightly retards peptide hydrogen exchange. The exchange curves of the unliganded pyridoxal form and its adsorption complex converge from an initial difference, whereas the exchange curve of the unliganded pyridoxamine form and its adsorption complex retain their small difference throughout the experiment.

The exchange behavior is markedly altered when, in the presence of both substrates, the enzyme is catalyzing the bond rearrangement steps of transamination. In the equilibrium mixture containing all covalent (and noncovalent) transamination intermediates (Fig. 1C), exchange is retarded 6 times, e.g., 220 hydrogens have exchanged at 225 min instead of at 40 min as in the unliganded pyridoxal form (Fig. 1A). The persistence of the vertical difference between the exchange curves of the unliganded enzyme and that of the covalent intermediates over the entire time course examined suggests that both rapidly and more slowly exchanging peptide hydrogens are affected. As a corollary, the total number of peptide hydrogens retarded in their exchange by formation of the covalent intermediates may be expected to be larger than apparent from the distance between the two curves (18). This is directly documented by an experiment in which the substrates were added to the unliganded pyridoxal form after initiation of the exchange reaction (intraexchange conversion, Fig. 2). Addition of the substrates decreases the rate of exchange such that between 1 and 6 hr only seven peptide hydrogens exchange instead of the 17 in the free pyridoxal form, 10 hydrogens being retarded by the conversion. Thus, the number of hydrogens affected in their exchange rate by the formation of the covalent intermediates is equal to the difference of 16 hydrogens manifest at the time of intraexchange conversion plus the 10 hydrogens shown to be retarded by the conversion, i.e., to about 26 hydrogens or 6% of the total 411 peptide hydrogens per subunit. Analogous results were obtained by intraexchange conversion of the unliganded pyridoxamine form of the enzyme into the covalent intermediates.[†] Even this figure may constitute an underestimate of the extent of the structural change caused

[†] The *intra*-exchange conversion of both unliganded enzyme forms into the corresponding nonproductive adsorption complexes by addition of the substrates after 50 min of exchange did not noticeably alter their exchange curves, thus confirming the similarity of the conformations of all noncovalent forms. Moreover, conversion of the pyridoxamine form into the nonproductive adsorption complex of the pyridoxal form by addition of 2 mM 2-oxoglutarate was without effect on the exchange reaction (14).

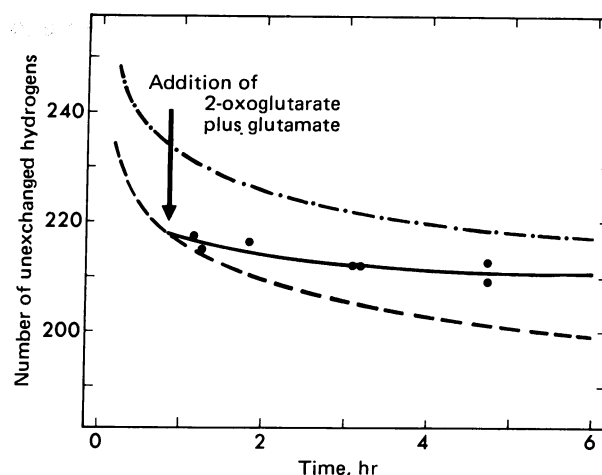


FIG. 2. Intraexchange conversion of the unliganded pyridoxal form into the covalent intermediates. Conditions were the same as in Fig. 1. At zero time, lyophilized enzyme in the pyridoxal form was dissolved in D₂O. After 50 min of exchange, 2 mM 2-oxoglutarate plus 70 mM glutamate was added by dissolving the appropriate quantity of lyophilized substrates (prepared as described in Fig. 1) in 500 μ l of the enzyme solution. The exchange data were obtained from two separate experiments. For comparison, the exchange curves of the pyridoxal form (---) and the covalent intermediates (-·-) are shown.

in the protein, because under the conditions employed only a minor fraction of the protein peptide groups is monitored.

DISCUSSION

A reduction in the rate of peptide hydrogen exchange as found in the present instance denotes a reduction in the exposure of the amide groups of the protein to the solvent. As pointed out for the couple oxyhemoglobin/deoxyhemoglobin, such changes can be visualized as arising from a restriction of intramolecular motions associated with the known conformational transitions of this system (19). Thus, the net retardation brought about in aspartate aminotransferase on formation of the covalent enzyme-substrate intermediates can be interpreted as a general tightening of enzyme structure.

The data obtained with aspartate aminotransferase may be interpreted as follows: The similarity of the exchange curves of the unliganded pyridoxal and pyridoxamine forms suggests similarity in their conformation. On formation of the adsorption complex of the pyridoxal enzyme with the substrate, as deduced from the retardation of some of the faster exchanging hydrogens in the pyridoxal enzyme-2-oxoglutarate complex, there is a relatively small change in conformation involving mainly peptide groups localized in the more exposed surface region of the molecule. Once the enzyme enters the covalent phase of catalysis, the conformational changes become much more pronounced and, as judged by the retardation of both faster and more slowly exchanging hydrogens, they now involve both superficial and more interior regions of the protein. On leaving the covalent phase to form the adsorption complex of the pyridoxamine form, the protein structure relaxes to a conformation which is similar to that of the free enzyme forms.

The succession of conformational changes along the reaction pathway of enzymic transamination concurs well with the structural deductions made from previous studies on the chemical modification of the thiol group of cysteine-390 in the same enzyme (6). In analogy to the striking effect on the hydrogen exchange rate, the reactivity of this residue towards sulfhydryl reagents changes by a factor of 100 under conditions

in which the covalent intermediates are formed; formation of the adsorption complexes, which also has a much smaller effect on the peptide hydrogen exchange, entails only a 2-fold change in rate. The two phenomena differ, however, in that the retardation of the hydrogen isotope exchange denotes an overall decrease in the reactivity of peptide hydrogens during substrate binding and catalysis, while cysteine-390 becomes in fact more reactive, thus testifying to the complexity of the syncatalytic conformational response.

The chemical nature of the covalent intermediates that give rise to exchange retardation and to the rate enhancement of cysteine-390 modification is not as yet identified. Because the substrate analogs 2-methylaspartate and *erythro*-3-hydroxyaspartate, though forming covalent intermediates (20, 21), exert only a very small effect on hydrogen exchange (14), it appears that the conformational response is highly specific and is linked to intermediate states that are formed or are populated extensively only in the transamination of the natural substrates. Hydrogen-deuterium exchange studies alone of course cannot give information on the detailed nature and localization of the syncatalytic conformational changes. From recent studies on the quaternary structure of aspartate aminotransferase it is known, however, that such effects do not result in functionally significant subunit interactions (22, 23); hence, it may be concluded that they do not reach the subunit interface but might be concentrated to the region surrounding the active site.

The tightening of protein structure during the bond rearrangement steps denotes a displacement of conformational equilibria in the protein and hence a difference in the conformational free energy content of unliganded aspartate aminotransferase and the covalent intermediates.[‡] Because this free energy increment can only derive from the interaction of the enzyme with its substrate, the observed effects document a partitioning of the chemical potential of the enzyme-substrate intermediates between the reaction center and the macromolecular matrix of the catalyst. While the precise delineation of the energetic coupling of the bond rearrangement steps with the conformational transitions will depend on the availability of high-resolution structural data, the present study lends experimental support to current concepts of a dynamic participation of the enzyme structure in catalysis (for reviews see refs. 2 and 4). Syncatalytic conformational changes might play an

important role in optimizing the free energy profile of the reaction by serving a "free energy buffer" function and/or by adjusting the topochemistry of the active site for the successive catalytic steps, and might thus contribute to the efficiency and specificity of enzymic transamination.

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[‡] The 6-fold retardation of exchange in the enzyme undergoing catalysis (Fig. 1C) relative to that of the unliganded pyridoxal form (Fig. 1A) corresponds to a free energy change of about 1 kcal/mol of subunit. As pointed out previously (24), this figure is a *minimum* value applying only to the unlikely case that the formation of the covalent intermediates uniformly retards the exchange of all peptide hydrogens monitored. To the extent that this condition is not met and that exchange retardation may in fact be masked by compensatory exchange acceleration in other parts of the molecule, the free energy increment associated with the syncatalytic conformational changes will be larger.

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