Crystalline aspartate aminotransferase: Lattice-induced functional asymmetry of the two subunits
(activity of crystalline enzyme/microcrystals/crystal effects/polyethylene glycol)

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ABSTRACT The enzymic activity of crystalline mitochondrial aspartate aminotransferase (L-aspartate:2-oxoglutarate aminotransferase, EC 2.6.1.1) was determined in suspensions of non-crosslinked microcrystals in 30% (wt/vol) polyethylene glycol. The crystals (average dimensions, 22 x 5 x 0.8 μm) were small enough to preclude diffusional rate limitation. They had the same habit as the triclinic crystals used for the determination of the spatial structure of the enzyme by x-ray crystallographic analysis [Ford, G. C., Eichele, G., and Jansonius, J. N. (1980) Proc. Natl. Acad. Sci. USA 77, 2559-2563]. Determination of the Michaelis-Menten parameters showed that the packing of the enzyme dimer into the crystal lattice not only decreases its activity but also induces a functional nonequivalence of the two subunits that behave identically in solution. The crystalline enzyme possesses a high-affinity subunit with K_m values similar to those of the enzyme in solution (K_m = 0.5 mM for aspartate and 1.2 mM for 2-oxoglutarate) and a low-affinity subunit (K_m = 5.5 mM and 14.5 mM, respectively). The catalytic activity of the high-affinity subunit is 3% and that of the low-affinity subunit is 15% of the activity of the enzyme in solution. The functional asymmetry of the crystalline enzyme dimer could also be demonstrated by selective mechanism-based modification of either type of active sites. In view of the apparently identical conformation of the two subunits in the crystalline enzyme, its decreased catalytic efficiency and its functional asymmetry likely are due to constraints exerted by the crystal lattice on the conformational adaptability of the two subunits. In triclinic crystals the two subunits of the enzyme dimer have dissimilar lattice contacts.

The purpose of this study was to compare the functional properties of crystalline mitochondrial aspartate aminotransferase (AspNTase; L-aspartate:2-oxoglutarate aminotransferase, EC 2.6.1.1) with those of the enzyme in solution. The differences found will have to be considered in deducing a detailed catalytic mechanism from the model of the enzyme’s spatial structure that has been provided by x-ray crystallographic analysis (1). In a crystal, lattice effects might interfere with the catalytic processes either by altering the conformation of the enzyme or by restricting its conformational adaptability.

Since the pioneering work of Richards and co-workers that showed crystals of RNase S (2) and carboxypeptidase A (3) to be enzymically active, the catalytic activities of several other enzymes in the crystalline state have been reported. Extensive studies have been performed on carboxypeptidases A (4, 5) and B (6), lactate dehydrogenase (7), and subtilisin (8). All investigations used microcrystals that were either crosslinked or kept insoluble in highly concentrated salt solutions (for reviews, see refs. 9 and 10).

In the case of crystalline AspNTase the catalytic activity measured in suspensions of noncrosslinked microcrystals was found to be 1 order of magnitude lower than that of the enzyme in solution (11). The decrease in molar activity is not due to rate limitation by diffusion of substrates and products in the liquid channels of the crystals. The absence of diffusional rate limitation in the microcrystals (average thickness, 0.8 μm) was verified by kinetic tests developed for the assessment of the kinetic behavior of immobilized enzymes (12, 13). Because the crystals were kept in 30% (wt/vol) polyethylene glycol (PEG) which does not penetrate the interior of the crystals (14), any deviating functional properties of the crystalline enzyme can be attributed unequivocally to crystal packing effects.

In the case of an oligomeric enzyme, such as AspNTase, the symmetry properties of the crystal may entail dissimilar sets of lattice contacts of intrinsically equivalent subunits. This situation holds for the triclinic crystals used in the structural and kinetic studies of AspNTase, in which the two subunits have a different environment (1). The possibility exists that the dissimilar lattice contacts induce a functional nonequivalence of the two subunits. In solution, the two subunits of AspNTase are functionally equivalent by manifold criteria (15-20). Microspectrophotometry of single crystals has shown that both active sites of the crystalline enzyme dimer can undergo the half-reactions of transamiation. The absorption spectra of the coenzyme chromophore in the different functional states of the enzyme are almost identical with those of the enzyme in solution (21-23). In crystals of unliganded AspNTase, no difference in conformation of the two subunits is detectable by x-ray crystallographic analysis at 2.8-A resolution (1). However, diffusing N-(5'-phosphopyridoxyl)-L-aspartate, a coenzyme-substrate analog, into crystals of apoenzyme induced a marked conformational change in only one subunit (24, 25), suggesting a lattice-induced difference in the conformational adaptability of the two subunits.

This study shows that the packing of the AspNTase dimer into the crystal lattice not only decreases its activity but also results in a functional nonequivalence of the two subunits.

EXPERIMENTAL PROCEDURE

Mitochondrial AspNTase was isolated from chicken heart (26). The specific activity of the enzyme preparations was ~200 units/mg. Microcrystals were grown in 15% (wt/vol) PEG (unpublished data). PEG 6000, synthetic grade, was purchased from Merck. The activity of the enzyme in solution was assayed photometrically by using the coupled test (27) with malate dehydrogenase (Boehringer) in 50 mM aspartate/50 mM 2-oxoglutarate/50 mM sodium phosphate/0.4 mM NADH, pH 7.5, at 30°C. The purpose of this study was to compare the functional properties of crystalline mitochondrial aspartate aminotransferase (AspNTase; L-aspartate:2-oxoglutarate aminotransferase, EC 2.6.1.1) with those of the enzyme in solution. The differences found will have to be considered in deducing a detailed catalytic mechanism from the model of the enzyme’s spatial structure that has been provided by x-ray crystallographic analysis (1). In a crystal, lattice effects might interfere with the catalytic processes either by altering the conformation of the enzyme or by restricting its conformational adaptability.

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Abbreviations: AspNTase, aspartate aminotransferase; PEG, polyethylene glycol; PEG solution, 30% (wt/vol) PEG in 50 mM sodium phosphate at pH 7.5.

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25°C. The activity of suspensions of noncrosslinked microcrystals of AspNTase was determined by the above test in the presence of 30% (wt/vol) PEG in 50 mM sodium phosphate at pH 7.5 (hereafter designated PEG solution). All assay suspensions were continuously stirred with a motor-driven Flexiglas helix dipped into half of a thermostated (25°C) cuvet; the light beam passed through the other half. The measured activity was independent of the stirring rate—i.e., it was not influenced by the Neinstein diffusion layer effects at the solution/crystal interface (28). The assay results were linear with time and enzyme concentration. The activity of the microcrystals was obtained by subtracting the activity of the supernatant of the crystal suspension from the total activity measured (centrifugation with a swing-out rotor at 54,000 × g, 20 min, 25 ± 2°C). Depending on the concentration of the crystal suspension, the correction was 5–20% of the total activity.

Protein concentrations were determined photometrically after dissolution of the crystals by using the extinction coefficient ε280 = 1.4 × 10⁵ M⁻¹ cm⁻¹ for the enzyme dimer. Alternatively, the activity of the dissolved enzyme was taken as a measure of its concentration. The pH value of buffered solutions was adjusted after the addition of PEG. For reduction of the internal Schiff base (29) in suspensions of microcrystals, two portions of a 100 mM stock solution of sodium borohydride in 50 mM NaOH/30% (wt/vol) PEG were added after 1 and 2 min to give a final concentration of 5 mM. Excess borohydride was trapped by adding excess pyridoxal 5'-phosphate.

RESULTS AND DISCUSSION

Determination of the rate of transamination in a suspension of microcrystals of AspNTase as a function of substrate concentration revealed a biphasic behavior indicating the existence of two populations of active sites differing in their Km values by about 1 order of magnitude (Fig. 1). The Km values of the high-affinity site for 2-oxoglutarate and aspartate are practically the same as those of the enzyme in solution (Table 1). The contribution of the high- and low-affinity sites to the activity of the crystals are 1.5 and 7.5%, respectively, of that of the enzyme in solution. If two different active sites in equimolar concentration are assumed (see below), their molar activities are 3% and 15% of those in solution. The previously measured overall activity per subunit of the crystalline enzyme (10% of that of the enzyme in solution) closely corresponds with the mean of the contributions of the two subunits.

A biphasic kinetic behavior with similar Km values was also found with crosslinked microcrystals of AspNTase (data not shown). Although the catalytic activity depends on the conditions used for crosslinking, the ratio of the activities of the high- and low-affinity sites of crosslinked crystals is the same as for the noncrosslinked crystals. The results with crosslinked crystals validate the assay procedure used for the noncrosslinked crystals that involves a centrifugation step in order to correct for the activity of dissolved enzyme.

The kinetic nonequivalence of the subunits of the crystalline enzyme contrasts with the behavior of the enzyme in solution where both subunits of the dimer are equivalent. The enzyme in solution shows a strictly monophasic dependence of its activity on the concentrations of both 2-oxoglutarate and aspartate (Km and Vmax values are given in Table 1). Detailed studies with the enzyme in solution, such as the determination of the activity of hybrid dimers with one active and one inactive subunit (15, 20) and the determination of the active site occupancy pattern at transamination equilibrium (30), gave no indication of a functional nonequivalence of the subunits or of functionally significant subunit interactions.

Like AspNTase in solution, the pyridoxal form of the crystalline enzyme is readily inactivated by reducing the Schiff base between lysine-258 and pyridoxal 5'-phosphate with sodium borohydride (29). Addition of cysteine sulfinate (final concentration, 5 mM) to the crystal suspension completely prevented borohydride from abolishing the enzymatic activity as measured both before and after dissolution of the crystals. Apparently, all active sites in the crystals had undergone the conversion to the pyridoxamine form. Addition of a keto acid (25 mM 2-oxoglutarate or 1 mM oxalacetate) to the washed suspension of microcrystals in the pyridoxamine form rendered them again susceptible to complete inactivation by sodium borohydride. After two additions of borohydride, the activity of both the crystals and the dissolved crystals was decreased to 2–5% of the initial values. These experiments provide chemical support for the conclusion reached previously from spectroscopic evidence (21) that all active sites in the crystals are catalytically competent.

The interpretation of the biphasic kinetic behavior to indicate two nonequivalent active sites in the crystalline enzyme dimer is corroborated by selective mechanism-based modification of either type of active sites. To a suspension of microcrystals in the pyridoxamine form, oxalacetate was added in substoichiometric amounts such that only a fraction of all subunits was converted to the pyridoxal form. At the same time,
excess sodium borohydride was added (Scheme 1). The subunits undergoing transamination faster under these conditions will be inactivated preferentially by reduction of the newly formed internal aldimine. The preferential inactivation of the “fast” subunits manifests itself in a greater decrease in the activity of the crystals than in the activity measured after dissolution of the crystals. In the crystals, the “fast” subunits contribute to a higher degree to the measured activity whereas in solution all active sites are equivalent (Fig. 2 Left).

If the assumption of two different subunits is correct, the converse experiment (Scheme 2) may be expected to result in the preferential inactivation of the “slow” subunits. Addition of limiting amounts of cysteine sulfinate to microcrystals in the pyridoxal form preferentially converts the “fast” subunits to the pyridoxamine form and thus preserves them from inactivation by subsequent addition of borohydride. The preferential escape of the “fast” subunits from inactivation is reflected in a smaller loss of activity of the crystals than of the dissolved crystals (Fig. 2 Right).

There are three possible explanations for the occurrence of functionally nonequivalent active sites in the suspension of microcrystals. (i) Two different populations of microcrystals could exist in the suspension. This possibility is excluded by two lines of evidence. Scanning electron microscopy showed that all microcrystals were of the same habit. X-ray analysis of larger crystals grown under the same conditions showed that they were all isomorphous (triclinic unit cell; J. N. Jansonius, personal communication).

(ii) In a crystal, two different populations of enzyme dimers could exist. A uniform distribution of two different dimers

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**Fig. 2.** (Left) Preferential inactivation of “fast” subunits. The plot combines three experiments. Microcrystals (pyridoxamine form, subunit concentration 0.1–0.4 μmol/ml of suspension) were kept in PEG solution. Sodium borohydride (100 mM stock solution in 50 mM NaOH; final concentration, 5 mM) and oxalacetate in the indicated concentrations were added simultaneously. The enzymic activity of both crystals and dissolved crystals were assayed at 150 mM aspartate and 6 mM 2-oxoglutarate. At the low concentrations of oxalacetate used, the subunit with the lower $K_m$ would be expected to react faster—i.e., to be inactivated preferentially. This subunit has the lower molar activity (Table 1) and is expected to contribute to a lesser degree to the total activity of the crystalline enzyme measured at high concentrations of substrates. Thus, in contrast to the experimental finding, the activity of the crystals would be expected to decrease less than the activity in solution. The apparent discrepancy must be due to the different conditions used: low concentration of oxalacetate as single substrate in a one-turnover reaction vs. high concentrations of an amino acid/keto acid substrate pair under steady-state conditions. (Right) Preferential escape of “fast” subunits from inactivation. The plot combines three experiments. Microcrystals (pyridoxal form, subunit concentration 0.1–0.4 μmol/ml of suspension) were kept in PEG solution. Cysteine sulfinate was added at the indicated concentrations. After 1 min, sodium borohydride was added (final concentration, 5 mM). The enzymic activities were assayed as indicated Left.
throughout the crystal is excluded because in our triclinic crystals the unit cell contains only one enzyme dimer (24)—i.e., in the crystal lattice each dimer has the same surroundings. The possibility that enzyme subunits at the surface of the crystals are less affected in their kinetic properties than those fully incorporated in the crystal lattice appears to be excluded by the following consideration. The average thickness of the crystals comprises about 80 layers of enzyme dimers (24). Because the dimers are arranged with their longest dimension nearly perpendicular to the main faces of the crystals (24, 25), the two main surfaces are formed by one type of subunit only [S or S*, respectively (1)]. Thus, the subunits of one of the two surface layers could account for a fraction of the measured activity corresponding at most to 1/160—i.e., to 0.6%—of the activity of the enzyme in solution. The molar activities of the high-affinity active sites whose $K_m$ is the same as that of the enzyme in solution (Table 1) is 1.5%, however.

(iii) The best explanation of the present data assumes that in the crystal the two subunits of the enzyme dimer have become kinetically different from each other. This idea is supported by x-ray crystallographic data. The two subunits obey a molecular dyad; however, they differ in their lattice contacts (24). Apparently, the different surroundings give rise to different kinetic properties of the two subunits that are functionally equivalent in solution and conformationally identical in x-ray analysis of the unliganded enzyme (1). A similar lattice-induced asymmetry has been reported for the reaction of ferrihemoglobin with azide that is monophasic in solution and becomes biphasic in the crystal. This finding has been explained by lattice-induced functional asymmetry of the subunits of the hemoglobin tetramer (31).

How does the asymmetric packing give rise to the observed functional asymmetry of the AspNTase dimer? The dissimilar surroundings either might result in a small difference in conformation that is not detected by x-ray analysis at the present level of resolution or might induce a difference in the conformational adaptability of the two subunits. A lattice-induced asymmetry in the conformational adaptability of the two subunits has been found by x-ray crystallographic analysis of the phosphopyridoxyl aspartate apoenzyme complex. Introduction of N-(5'-phosphopyridoxyl)-L-aspartate, an analog of the covalent coenzyme–substrate intermediates, into apoenzyme crystals that are isomorphous with holoenzyme crystals led to a marked conformational change in all but one of the two subunits (24, 25). The coincidence of low and high affinities for the substrate with high and low molar activities, respectively (Table 1), suggests that, in the low-affinity subunit, part of the binding energy of the substrate is used for a conformational change, resulting in a higher reaction rate of this subunit.

The present study emphasizes the importance of a detailed kinetic analysis for the assessment of lattice effects on the functional properties of crystalline enzymes. Prerequisites for obtaining significant data (11) are: (i) exclusion of diffusional rate limitation; (ii) use of fast-reacting natural substrates; and (iii) use of a crystallization medium not directly interacting with the enzyme, a condition met by PEG of higher molecular weight that does not penetrate the interior of the crystals (14, 32).

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