Catalytic activity of non-cross-linked microcrystals of aspartate aminotransferase in poly(ethylene glycol)

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The molar activity of crystalline mitochondrial aspartate aminotransferase is decreased to 10% of that of the enzyme in solution. The activity was measured in suspensions of non-cross-linked microcrystals (average dimensions $22\mu\text{m} \times 5\mu\text{m} \times 0.8\mu\text{m}$) in 30% (w/v) poly(ethylene glycol). Kinetic tests ruled out the possibility that diffusion of the substrate in the crystals is rate-limiting. The observed decrease in catalytic efficiency can be attributed exclusively to crystal-packing effects. A direct inhibition by poly(ethylene glycol) is excluded because poly(ethylene glycol), with average M_r 6000, cannot penetrate the liquid channels of the crystals, owing to its large Stokes radius. The crystals examined were triclinic and of the same habit as those used for high-resolution X-ray-crystallographic analysis [Ford, Eichele & Jansonius (1980) *Proc. Natl. Acad. Sci. U.S.A.* 77, 2559–2563]. The catalytic competence of crystalline aspartate aminotransferase confirms the relevance of the spatial model of this protein for the elucidation of its mechanism of action.

Recently, X-ray-crystallographic analysis in conjunction with the determination of the amino acid sequence (Barra *et al.*, 1980; U. Graf-Hausner, K. J. Wilson & P. Christen, unpublished work) has yielded a model of the spatial structure of mitochondrial aspartate aminotransferase (AspAT) at 0.28 nm (2.8 Å) resolution (Ford *et al.*, 1980). The crystallographic analysis was performed with triclinic crystals that were grown in poly(ethylene glycol) (PEG) and contain one enzyme dimer per unit cell. Microspectrophotometric analysis of single crystals showed that both active sites of the AspAT dimer are catalytically competent. On soaking in 20% (w/v) PEG solutions containing the appropriate substrate, the enzyme crystal underwent either half-reaction of transamination without having impaired its crystalline order (Eichele *et al.*, 1978). Because of the diffusional rate limitation in large-sized crystals, the catalytic activity of crystalline AspAT could not be determined quantitatively by microspectrophotometry. An estimate of the catalytic efficiency of the crystalline enzyme is essential, however, in deducing a detailed mechanism of action from the model of the spatial structure as determined by X-ray crystallography. In the present study, diffusional rate limitation was precluded by

measuring the catalytic activity of AspAT in suspensions of microcrystals. To date, only a few crystalline enzymes have been examined in this way [for reviews, see Rupley (1969) and Makinen & Fink (1977)]. More recent studies were reported on lactate dehydrogenase (Bayne & Ottesen, 1976), carboxypeptidase A (Spilburg *et al.*, 1977), carboxypeptidase B (Alter *et al.*, 1977) and subtilisin (Tüchsen & Ottesen, 1977). These previous investigations were performed with microcrystals that were either covalently cross-linked or kept insoluble in highly concentrated salt solutions. In the case of AspAT, PEG at 30% (w/v) concentration decreased the solubility of the enzyme sufficiently for dispensing with cross-linking the microcrystals. Thus any interference of uncontrolled modifications of the crystalline enzyme with its catalytic action was avoided. In contrast with salt ions or organic solvents of other crystallization media, PEG of high M_r (~6000 in the present study) cannot penetrate the interior of the crystals because of its random-coil behaviour, giving it a large Stokes radius (McPherson, 1976; Ingham, 1977). The liquid channels surrounding the enzyme molecules in the crystal and giving the substrates access to the active sites are filled with buffer solution only. Any difference in the catalytic efficiency between the crystalline enzyme and the enzyme in solution must be due to crystal-packing effects and not to direct effects of the crystallization medium.

Abbreviations used: AspAT, aspartate aminotransferase; PEG, poly(ethylene glycol) 6000; PEG solution, 30% (w/v) poly(ethylene glycol) 6000/50 mM-sodium phosphate, pH 7.5.

Experimental procedure

Materials

Mitochondrial AspAT was isolated from chicken heart as described previously (Gehring *et al.*, 1977a). All chemicals used were of the highest available purity. PEG 6000 for synthetic purposes was purchased from Merck. DL-Vinylglycine was a gift from Dr. R. R. Rando, Harvard Medical School, Boston, MA, U.S.A. The pH values of buffered solutions were adjusted after the addition of PEG. PEG at 30% (w/v) shifts the pH value of 50 mM-sodium phosphate, pH 7.5, by 0.25 unit toward the alkaline (Eichele *et al.*, 1978; Atha & Ingham, 1981).

Assay for AspAT activity in solution

The photometric assay coupled with malate dehydrogenase (EC 1.1.1.37; from pig heart; purchased from Boehringer) was performed as described by Karmen (1955) in 50 mM-aspartate/50 mM-2-oxoglutarate/50 mM-sodium phosphate/0.4 mM-NADH/malate dehydrogenase [1.6 units ($\mu\text{mol}/\text{min}/\text{ml}$), pH 7.5 at 25°C. The reaction was started by addition of 5–50 μl of AspAT (final concn. 0.01–0.1 unit/ml) to 2.990 ml of the assay solution. In an alternative assay not depending on malate dehydrogenase, the reaction was monitored by the absorption of oxalacetate generated (ϵ_{280} 499 $\text{M}^{-1}\cdot\text{cm}^{-1}$; Nisonoff *et al.*, 1952) in the same assay solution without NADH and malate dehydrogenase.

Assay for alanine aminotransferase activity in solution

The assay solution was 400 mM-alanine/6 mM-2-oxoglutarate/50 mM-sodium phosphate/0.6 mM-NADH/lactate dehydrogenase (14 units/ml; EC 1.1.1.27; from rabbit muscle; purchased from Boehringer), pH 7.5, at 25°C. The reaction was started by addition of 10 μl of AspAT (~10 units of AspAT activity/ml of assay solution) to 1.045 ml of the assay solution. The apparent activity was corrected for NADH consumed in the reduction of 2-oxoglutarate catalysed by lactate dehydrogenase (Holbrook *et al.*, 1975).

Protein determination

Protein concentrations in suspensions of microcrystals were determined after dissolution of the crystals in 50 mM-sodium phosphate, pH 7.5, either spectrophotometrically (ϵ_{280} 1.4 $\times 10^5$ $\text{M}^{-1}\cdot\text{cm}^{-1}$ for the enzyme dimer) or by measuring the enzymic activity.

Results and discussion

Crystallization

To a solution of AspAT (5–8 mg/ml) in 50 mM-sodium phosphate, pH 7.5, 0.2 mM-pyridoxal 5'-

phosphate was added. After 20 min at room temperature and addition of 2 mM-2-oxoglutaric acid, the solution was passed through a Sephadex G-25 column that was eluted with 50 mM-sodium phosphate, pH 7.5. The enzyme solution was concentrated to 10 mg/ml by ultrafiltration (PM 10 membrane from Amicon); the specific activity was 200 units/mg.

Crystallization was performed in a plastic test tube (60 mm $\times$ 9 mm) equipped with a magnetic stirring bar and kept at 25°C in a water bath. To the concentrated enzyme solution (300 μl) an equal volume of 30% (w/v) PEG/50 mM-sodium phosphate, pH 7.5 (PEG solution) was added dropwise with continuous stirring. The solution became turbid during the addition of the last drops of the PEG solution. Within 2 min, the first crystals were discernible under the optical microscope (magnification $\times 70$). After 5 min, the crystals had reached an average length of 22 μm . The crystals were transferred into PEG solution by centrifuging twice at 1500 g in a swing-out rotor for 1 min at room temperature and suspending the sediment in 300 μl of PEG solution. In order to inhibit microbial growth, NaN_3 (0.2 mg/ml) was added.

Fractionation of microcrystals

Five suspensions (300 μl each) obtained as described above were combined and subjected to the following treatment: (1) The tube (60 mm $\times$ 9 mm) containing the crystal suspension was sealed with Parafilm and rotated end-over-end at 2 rev./min and room temperature in the dark for 5 h. Apparently, this procedure resulted in a reorganization of part of the crystals (see under 'Saturation concentration of AspAT in PEG solution' below). (2) The tube was kept at 4°C in the dark until a loose sediment had been formed after 1 to 3 days. (3) The supernatant containing very small crystals and some amorphous material was removed with a pipette. The sediment was suspended in the same tube with 700 μl of PEG solution. After 1 h at room temperature the tube was centrifuged at 200 g for about 10 s. (4) The supernatant was transferred into another tube and centrifuged at 1500 g for 90 s. The sediment was suspended in PEG solution to give an enzyme concentration of 5 mg/ml. After a new addition of NaN_3 the crystal suspensions were stored in the dark at 4°C.

Crystallization of thicker microcrystals

The first steps were the same as those described under 'Crystallization' above. However, after formation of the microcrystals, 150 μl of PEG solution were added anew. Subsequently, 300 μl of enzyme solution (10 mg/ml) were added dropwise (within 2 min) under continuous slow stirring. The magnetic rod was then removed and the tube was subjected to

the slow end-over-end rotation described above for 45 min. Subsequently the PEG concentration in this suspension (13%, w/v) was increased to 17% by adding $3 \times 100 \mu\text{l}$ of PEG solution in intervals of 15 min. In between, the tube was continuously rotated. The crystals were transferred into PEG solution by two subsequent centrifugations as described under 'Crystallization' above. After rotating the tube for 5 h, it was kept at room temperature for 30 min until a sediment began to form. After centrifugation at 200 g for ~15 s the sediment was suspended in 1 ml of PEG solution and the centrifugation was repeated. The sediment was suspended in PEG solution and azide was added.

Scanning electron microscopy

A sample of the crystal suspension ($10 \mu\text{l}$) was added to $50 \mu\text{l}$ of PEG solution containing 1.8% (w/v) glutaraldehyde and kept at room temperature for 30 min. The cross-linked crystals were suspended in 0.5 ml of water and applied on to a Nuclepore filter (from Nuclepore Corp., Pleasanton, CA, U.S.A.; pore diameter $1 \mu\text{m}$) that had been positioned on a sintered-glass funnel using slightly reduced pressure (53 kPa). The crystals were dehydrated by a series of ethanol/water mixtures with

increasing ethanol content: 50, 70, 80, 90, 96% (v/v). In each solution the crystals were kept for 10 min, the last step being repeated. The crystals were dried in a sputtering device (Balzers-Union, Balzers, Liechtenstein) at 6.7 Pa for 10 min and sputtered with gold under argon (13.4 Pa). On inspection with a scanning electron microscope (Cambridge S4) the crystals proved to be of fairly uniform size (Fig. 1). Determination of the angles between the edges showed the microcrystals to be of the same habit as the large crystals used for X-ray-crystallographic analysis.

Saturation concentration of AspAT in PEG solution

The saturation concentration of the enzyme was determined under assay conditions. Concentrated stock suspensions of crystals were transferred into the substrate containing PEG solution (assay PEG solution; the composition is indicated in legend of Fig. 2). At timed intervals the concentration of the enzyme in the supernatant was determined (Fig. 2). The constant value reached after 5 to 7 h is independent of the total amount of microcrystals added and is assumed to correspond to the saturation concentration ($0.15 \mu\text{g/ml}$ of assay PEG solu-

Fig. 1. *Form and size of microcrystals of mitochondrial AspAT*

(a) Scanning electron micrograph (courtesy of Dr. U. Jauch and Dr. D. Metzger, Institut für Pflanzenbiologie, University of Zurich). The vertical bar indicates $50 \mu\text{m}$. A comparison of the length of very large crystals ($250 \mu\text{m} \times 30 \mu\text{m} \times 30 \mu\text{m}$) as measured in the native state under the optical microscope with the length of dehydrated cross-linked crystals as measured with the scanning electron microscope did not show significant differences. (b) Histogram of length of crystals as measured on scanning electron micrographs. The crystals were from three different crystallization batches. The average dimensions of the microcrystals used in the present study were $22 \mu\text{m} \times 5 \mu\text{m} \times 0.8 \mu\text{m}$ (s.d. $\pm 0.25 \mu\text{m}$; $n = 30$). The thickness was measured by tilting the object table by 90° .

tion). In the first 2–3 h a transient overshoot in non-sedimentable enzyme is observed that increases with increasing amount of microcrystals added. End-over-end rotation of the stock suspension

Fig. 2. Determination of the saturation concentration of AspAT by adding crystalline enzyme to assay PEG solution

The concentration of enzyme in the supernatant is given as a function of time at different total concentrations of microcrystalline AspAT: ▲, 1.5 mg/ml; ■, 0.07 mg/ml; ●, 0.03 mg/ml. Stock suspensions of freshly crystallized AspAT that had not been subjected to the end-over-end rotation procedure were concentrated by centrifugation to 4.5 and 43.5 mg/ml and transferred into 1.45 ml of PEG solution containing 150 mM-aspartate and 6 mM-2-oxoglutarate in a plastic test tube. The tube was sealed with Parafilm and continuously rotated end-over-end to prevent sedimentation of the crystals. In timed intervals, 100 μ l samples were centrifuged in a swing-out rotor at 54 000 g for 20 min at $25 \pm 2^\circ\text{C}$ (for details, see under 'Catalytic activity of crystalline AspAT' in the Results and discussion section). The concentration of the enzyme in the supernatant was determined by its activity in an assay without PEG. The inset shows the effect of preceding end-over-end rotation of the suspension of microcrystals. After rotating for 5 h the suspension (▲, 1.5 mg/ml) was diluted with assay PEG solution to 0.03 mg/ml (○). The experiment with non-rotated crystals at the same concentration (●) is replotted. The specific activity of the enzyme in the crystals, as measured in an assay without PEG, was unchanged at the end of the experiments.

significantly decreases the fraction of non-sedimentable enzyme (Fig. 2, inset). The fraction of enzyme in the supernatant exceeding the saturation concentration probably exists in the form of non-sedimentable metastable aggregates corresponding to maximally 2% of the total protein. The decrease in overshoot after end-over-end rotation of the stock suspension most likely is due to a reorganization of the most fragile crystals preventing physical damage of part of the crystals during transfer into the assay. Foltmann (1959) reported that the rate of dissolution of crystalline chymosin was diminished when crystals were used for a second time in solubility studies. Damage of the microcrystals due to substrate-induced conformational changes (Gehring & Christen, 1978; Eichele *et al.*, 1979) can be excluded because much larger crystals underwent transamination without having their crystalline order impaired (Eichele *et al.*, 1978). In the present study, newly crystallized AspAT was routinely subjected to end-over-end rotation (see under 'Crystallization' above).

The saturation concentration of AspAT was also estimated by adding dissolved AspAT to assay PEG solution. The ensuing enzymic activity was compared with that remaining in the supernatant after high-speed centrifugation (Fig. 3). Over the whole concentration range tested, the activity of AspAT in assay PEG solution was 40 to 50% of that in the absence of PEG. The activity increased as a linear function of enzyme concentration in the total range tested, indicating that the inhibiting effect of PEG is independent of the state of aggregation of the enzyme. At enzyme concentrations in excess of 0.15 μ g/ml, increasing amounts of activity were removed by centrifugation, thus confirming the above estimate of the saturation concentration. The sedimentable material generated above the saturation limit was an amorphous precipitate. Its activity increased about 2-fold on dissolution in PEG-free assay solution (Fig. 3). The amount of non-sedimentable material increased with increasing amounts of enzyme added, even above the saturation limit. This material probably represents small metastable aggregates of the same kind that account for the transient overshoot of non-sedimentable material in the experiment of Fig. 2. The fact that PEG decreases the enzymic activity of AspAT independent of its state of aggregation indicates that the inertness of PEG toward proteins, as reported in other cases (Amiconi *et al.*, 1977; Atha & Ingham, 1981), may not apply generally.

In the activity assay performed in the first few minutes after addition of the microcrystals, the fraction of non-sedimentable enzyme increases on the ascending limb of the overshoot peak (see Fig. 2, inset). Because the extent of the overshoot depends on the amount of crystalline enzyme added and

Fig. 3. Determination of the saturation concentration by adding dissolved AspAT to assay PEG solution

A constant volume (10 μ l) of an appropriately prediluted stock solution of AspAT was added to 1.47 ml of assay PEG solution to give the indicated final concentrations. The assay PEG solution contained 30% (w/v) PEG, 150 mM-aspartate, 6 mM-2-oxoglutarate, 0.4 mM-NADH and 2.5 units of malate dehydrogenase/ml (activity measured in PEG solution). The enzymic activity was determined immediately after the addition of the enzyme, before centrifugation (Δ) and in the supernatant after centrifugation (∇) at 54 000 g for 20 min at $25 \pm 2^\circ\text{C}$ (for details of the assay and the centrifugation, see the Experimental procedure section). As a measure of the enzyme concentration in the supernatant, the enzymic activity was determined in an assay without PEG ($\circ$) at the same substrate concentrations.

varies somewhat for the different batches of crystal suspensions used, the activity of the microcrystals was obtained by correcting the activity measured in each assay individually by the activity measured in the corresponding supernatant.

Catalytic activity of crystalline AspAT

The enzymic activity of suspensions of microcrystals was assayed in cuvettes with an optical pathlength of 0.5 cm (other dimensions 1.0 cm $\times$ 4.4 cm), thermostatically controlled at 25°C . On one side, a helical Plexiglass stirrer (diameter 0.4 cm, pitch 1.6 cm) was dipped into the cuvette; through the other side the light beam passed (slit width 4 mm in both the Eppendorf photometer and the Hitachi/Perkin-Elmer spectrophotometer, type 340). The stirring rate was 800 rev./min. The assays were performed in PEG solution containing the substrates aspartate and 2-oxoglutarate at the indicated

concentrations. To 1.40 ml of this solution, 20 μ l of NADH (final concn. 0.4 mM) and 50 μ l of malate dehydrogenase (final concn. 2.5 units/ml) were added. The activity of malate dehydrogenase was measured with stirring in 30% (w/v) PEG/0.5 mM-oxalacetate/0.2 mM-NADH/0.1 M-sodium phosphate, pH 7.5, 25°C (Bergmeyer, 1974). The measured activity was 30% of the activity (1200 units/mg) measured in the absence of PEG. The assays were linear with respect to time and enzyme concentration (2–20 μ g/ml of assay solution).

The assay of AspAT was started by addition of 5–10 μ l of a suspension of microcrystals with a capillary pipette (uniform diameter 1 mm). The reaction progress curves were linear until all the NADH had been consumed (20 min at low enzyme concentration). Routinely, the reactions were monitored for 3–5 min. Subsequently, the total assay suspension was transferred into a 1.5 ml polypropylene tube and centrifuged with a swing-out rotor (SW 27) in an ultracentrifuge (Beckman L5-65) at 54 000 g for 20 min. Centrifugation was started when the pressure had reached 0.07 Pa. Setting the temperature control of the centrifuge to 26°C , the temperature of the sample was kept at $25 \pm 2^\circ\text{C}$ as measured with a thermocouple after the centrifugation. The supernatant (1.3 ml) was transferred with a Pasteur pipette into a plastic test tube and mixed with newly added NADH and malate dehydrogenase (final concns. 0.4 mM and 2.5 units/ml respectively) on a vibratory mixer; its enzymic activity was then measured as described above. The new addition of malate dehydrogenase was necessary because about 40% was in the sediment. The activity of the microcrystals was obtained by subtracting the activity of the supernatant from the total activity measured in the crystal suspension. Depending on the amount of crystals in the assay, the correction was 5–20% of the total activity. The enzymic activity of suspensions of both thin and thick microcrystals (average thickness 0.8 μ m and 6.5 μ m respectively) was independent of the stirring rate from 700 to 3000 rev./min (at stirring rates in excess of 1600 rev./min the thicker crystals were cracked). Thus the measured activity is not influenced by Nernst diffusion-layer effects at the solution/crystal interface (Ginzburg & Katchalsky, 1963). In an alternative assay (the same as that described above for AspAT in solution), the reaction was monitored by the absorption of oxalacetate generated (ϵ_{280} 920 $\text{M}^{-1}\cdot\text{cm}^{-1}$). The higher absorption coefficient of oxalacetate in PEG solution (cf. Nisonoff *et al.*, 1952) is probably due to a shift in the oxo-enol equilibrium of oxalacetate.

The molar activity of microcrystalline AspAT measured at 50 mM-aspartate and 100 mM-2-oxoglutarate is 10% of that of the enzyme in solution

(Table 1). Both methods for monitoring the enzymic reaction in the crystal suspension, i.e. the coupled assay with malate dehydrogenase and direct photometric determination of oxalacetate formed, gave the same results.

Catalytic activity of cross-linked crystals

Microcrystals were cross-linked by treatment with 25 mM-glutaraldehyde in PEG solution for 5 s at room temperature. The reaction was stopped by diluting the suspension 10-fold with buffer containing no PEG. Subsequently, the suspension was centrifuged for 15 min at 1500 g. The sediment was resuspended in the same buffer. The activity measured in the supernatant of cross-linked microcrystals was less than 0.1% of the total activity. The enzymic activity of cross-linked microcrystals markedly depended on the extent of exposure to glutaraldehyde. In crystals cross-linked as described above it was 6% of that of the enzyme in solution, regardless of whether it was measured in the presence or absence of 30% (w/v) PEG. This finding supports the notion that PEG is excluded from the interior of the crystals (McPherson, 1976; Ingham,

1977). The catalytic activity of non-crystalline AspAT is decreased by 30% (w/v) PEG to about 50% of that of the uninhibited enzyme (Fig. 3).

Estimation of diffusional rate limitation in microcrystals

Concentration gradients of the substrates in the interior of the crystals arise when their diffusion in the liquid channels is slow in comparison with the reaction rate. Such concentration gradients will result in a lowered reaction rate in the central parts of the crystal, particularly at substrate concentrations near or below the K_m value of the enzyme. A quantitative theory elaborated by Katchalski *et al.* (1971) and by Laidler & Bunting (1980) describes the kinetic properties of enzymes immobilized in artificial matrices. An important part of these studies was the specification of experiments for assessing diffusional rate limitation in immobilized enzymes. Spilburg *et al.* (1977) applied the theory to carboxypeptidase A immobilized by crystallization. The concentration of the active sites in the crystals was varied with an irreversible inhibitor. If there is no rate limitation by diffusion of reactants, the catalytic activity will be a linear function of the concentration of active sites in the crystals. If the activity is limited by diffusion, it will become a function of the square root of the active-site concentration in the crystals.

For the estimation of the diffusional rate limitation of microcrystalline AspAT the active-site concentration, i.e., the concentration of active subunits in the microcrystals, was decreased with the enzyme-activated inhibitor vinylglycine. The irreversible inhibition is due to the enzyme-catalysed transformation of vinylglycine to the 2,3-unsaturated enamine and subsequent nucleophilic attack of the 6-amino group of the active-site lysine residue (Gehring *et al.*, 1977b). The reaction of vinylglycine with the crystalline enzyme was monitored by measuring the decrease in enzymic activity of the microcrystals (Fig. 4). The slow rate of reaction ($t_{1/2}$ 30 min) ensures a uniform distribution of the inactivated subunits throughout the interior of the crystals. Consistent with this assumption, the same relative decrease in enzymic activity of the modified crystals was measured in the crystal suspension and after dissolution of the crystals. The measured activity was taken to represent the concentration of the active subunits in the crystals.

The enzymic activity of microcrystals that had been inactivated with vinylglycine to different extents was measured at substrate concentrations below the K'_m (apparent K_m) values. For thin crystals (average thickness 0.8 μm) the activity proved to be a linear function of the concentration of active subunits in the crystals, indicating the absence of

Table 1. Catalytic activity of crystalline AspAT

Result (a) below shows the activity of thin microcrystals (average thickness 0.8 μm) as determined with the coupled test with malate dehydrogenase. The concentrations of aspartate and 2-oxoglutarate were 50 mM and 100 mM respectively. The measured activity was a linear function of enzyme concentration in the range from 1 to 55 $\mu\text{g}/\text{ml}$ (0.02–1.1 units/ml). Result (b) shows the activity of thin microcrystals determined by direct photometric measurement of oxalacetate formed. The substrate concentrations were the same as in (a). The measured activity was a linear function of enzyme concentration in the range from 1 to 33 $\mu\text{g}/\text{ml}$. For (c) the same activity was measured in suspensions of thin and thick microcrystals (average thickness 0.8 and 6.5 μm respectively). Result (d) was determined microspectrophotometrically with single crystals (thickness 10–30 μm). At zero time the crystal was immersed in 20% (w/v) PEG solution containing 400 mM-alanine. As soon as the crystal had been placed in the light beam (after 1–2 min) the decrease in A_{355} was recorded. The measurements were carried out at room temperature. Results in (e) were determined as described in the Experimental procedure section.

	Molar activity per subunit (min^{-1}) towards:	
	Aspartate	Alanine
Crystalline enzyme	(a) 900 (b) 990	(c) 0.3 (d) 0.1
(e) Enzyme in solution	9000	0.5

Fig. 4. Inactivation of microcrystalline AspAT by the enzyme-activated inhibitor vinylglycine

▲, Relative activity of microcrystals in assay PEG solution; ●, after dissolution in an assay without PEG. DL-Vinylglycine (19 mM) was added to a suspension of microcrystals (pyridoxal form; average thickness $0.8\ \mu\text{m}$; subunit concentration $0.16\ \mu\text{mol}$ per ml of suspension) in PEG solution containing 3.5 mM-2-oxoglutarate.

diffusional rate limitation (Fig. 5a). The validity of the test is demonstrated by the results obtained with thicker crystals (average thickness $6.5\ \mu\text{m}$), where the measured activity proves to be a linear function of the square root of the concentration of active subunits (Fig. 5b).

Activity toward alanine

The assay conditions were the same as for the enzyme in solution except that the assay suspension contained 30% (w/v) PEG. The assay solutions were stirred. Alternatively, the activity of single crystals was measured with a Zeiss UMSP I recording microspectrophotometer as described previously (Eichele *et al.*, 1978). The activity of the crystalline enzyme toward alanine is about the same as that of the enzyme in solution (Table 1). In solution the reaction with alanine is about 10^4 times slower than that with aspartate. Suspensions of both thick and thin microcrystals as well as large single crystals show about the same activity, i.e. there is no diffusional rate limitation with this slowly reacting substrate.

The failure of the experiments with alanine (and for that matter also with vinylglycine) to detect the altered enzymic properties of crystalline AspAT emphasizes the importance of the use of fast-reacting natural substrates for assessing the functional consequences of the incorporation of enzyme molecules into a crystal lattice.

Fig. 5. Estimation of diffusional rate limitation in thin and thick microcrystals

The concentration of active subunits was varied by the enzyme-activated inhibitor vinylglycine (see Fig. 4). The activity of the microcrystals was measured at substrate concentrations below K'_m , i.e. with 0.2 mM-aspartate (K'_m 0.5 mM) and 6 mM-2-oxoglutarate. A similar plot was obtained when the enzymic activity was measured at 0.2 mM-2-oxoglutarate (K'_m 1.2 mM) and 150 mM aspartate. Enzymic activities are expressed as percentages of the initial activity of unmodified thin crystals. The concentrations of active subunits are expressed as percentages of the initial value for the unmodified crystals as obtained from their activities measured after dissolution in an assay without PEG. (a) Dependence of the activity of thin (●, average thickness $0.8\ \mu\text{m}$) and thick (○, average thickness $6.5\ \mu\text{m}$) microcrystals on the concentration of active subunits. (b) The same enzymic activities plotted as a function of the square root of the concentration of active subunits.

Conclusions

The experimental conditions under which the enzymic activity of crystalline AspAT was measured ensures that any observed difference in the catalytic parameters from those of the enzyme in solution can be unequivocally attributed to crystal-packing effects. The use of non-cross-linked crystals obviates interference by modified side chains, the non-penetrating PEG ensures that the liquid channels in the interior of the crystals are filled with buffer only, and the thinness of the microcrystals allows activity to be measured without diffusional rate limitation.

How do lattice forces decrease the catalytic efficiency of the enzyme? Lattice forces must either distort the geometry of the active site or impede ligand-induced or syncatalytic conformational adaptations that are integral features of the catalytic mechanism (Gehring & Christen, 1978; Pfister *et al.*, 1978). A detailed kinetic analysis of crystalline AspAT in conjunction with mechanism-based chemical modifications has even indicated that its measured molar activity corresponds to the mean of two functionally non-equivalent active sites of the crystalline enzyme dimer. Apparently, lattice forces affect the two subunits of the enzyme dimer differentially (Kirsten *et al.*, 1983).

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