Catalytic Activity in Crystals of Mitochondrial Aspartate Aminotransferase As Detected by Microspectrophotometry

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SUMMARY
Triclinic single crystals of mitochondrial aspartate aminotransferase from chicken were examined by microspectrophotometry using nonpolarized light. The crystalline enzyme shows the same protein and coenzyme absorption bands in the 250 to 500 nm region as the enzyme in solution. The spectrum of the pyridoxal form ($\lambda_{\text{max}}$ 355 nm) changes into that of the pyridoxamine form ($\lambda_{\text{max}}$ 333 nm) when excess cysteine sulfinate is added to the medium (20% (w/v) polyethylene glycol 6000/50 mM sodium phosphate (pH 7.5)). Addition of excess oxalacetate reverses the spectral change. In either direction, the conversion is complete, indicating that both active sites of the enzyme dimer are catalytically competent in the crystal lattice. As determined by spectrophotometric pH titration of crystals, the apparent $pK_a$ of enzyme-bound pyridoxal-5'-P is 6.1 to 6.2, i.e. about the same as in solution. The changes of the absorption spectrum brought about by the addition of substrates and substrate analogs essentially correspond to those observed in solution. Thus, addition of 2-oxoglutarate to the pyridoxal form of the crystalline enzyme at pH 7.5 generates the 430 nm absorption band ascribed to a nonproductive adsorption complex. On addition of the substrate analog 3-hydroxyaspartate, the crystals turn red due to the intense absorption maximum at 495 nm of the semiquinoid intermediate. The different absorption bands of the enzyme substrate intermediates generated in the presence of 2-methylaspartate or of the transaminating substrate pair aspartate and oxalacetate ($\lambda_{\text{max}}$ 340, 362 (sh), 430 nm) differ in their relative intensities from those observed in solution.

Aspartate aminotransferase is the most extensively investigated representative of the large group of pyridoxal-phosphate-dependent enzymes functioning in amino acid metabolism. It thus is the favorite object for studies aiming at a description of protein-assisted pyridoxal catalysis at the atomic level. The enzyme is a dimer of identical subunits with a molecular weight of $2 \times 45,000$ (1). Recently, large crystals of the mitochondrial isoenzyme of aspartate aminotransferase from chicken were obtained by vapor phase diffusion from solutions in polyethylene glycol (2). The crystals proved suitable for x-ray crystallographic analysis, their diffraction pattern extending to at least 1.9 Å. They are triclinic (space group $P1$) and contain a single enzyme dimer per unit cell. Diffraction data to 4.5 Å resolution have been collected for the holoenzyme and five heavy atom derivatives. The electron density map clearly reveals the molecular boundary of the dimer with the monomers related by a 2-fold axis. Collection of x-ray data to high resolution and determination of the amino acid sequence are now in progress. Similar work on the cytosolic isoenzymes from chicken and pig is being pursued in other laboratories (4, 5).

Studies on the interaction between crystalline enzyme and substrate may be expected to link the crystallographic data with the functional and conformational features of the enzyme in solution. In the case of aspartate aminotransferase, the examination of the functional properties of the crystalline enzyme seems particularly attractive because changes in the absorbance of the coenzyme (see Ref. 1) allow easy detection of the catalytic events. This communication reports on the observation of transamination in single crystals of mitochondrial aspartate aminotransferase by microspectrophotometry.

EXPERIMENTAL PROCEDURES
Materials—The isolation and crystallization of mitochondrial aspartate aminotransferase from chicken heart have been reported elsewhere (2). For microspectrophotometry, crystal plates of average dimensions of $0.3 \times 0.2 \times 0.01$ to $0.03$ mm were selected. The pyridoxal and the pyridoxamine form of the enzyme in solution were prepared as described earlier (7). L-erythro-3-Hydroxyaspartic acid was synthesized from copper glycinate and glyoxylate (8). Polyethylene glycol 6000, pyridoxal-5'-phosphoric acid, and L-aspartic acid were purchased from Merck, 2-oxoglutaric acid and oxalacetic acid were from Fluka; L-cysteine sulfinate and 2-methyl-DL-aspartic acid were from Sigma.

Methods—A Zeiss UMSP I recording double beam microspectrophotometer with nonpolarized light was used to measure transmission spectra of the enzyme crystals. The transmission spectra were transformed into absorption spectra using the transmission of the medium surrounding the crystal as reference value. The dimensions of the measured area were 32 µm. This area is screened out just before the light beam enters the photomultiplier. The crystals, immersed in PEG solution containing the indicated reagents, were placed between two quartz slides, the upper one being supported by two cover slips. The pH values of the PEG solutions were adjusted after addition of PEG to the buffered solution. The composition of the medium was altered either by replacing the PEG solution while the crystal remained on the microscope slide or by transferring the crystal into a vial containing the new solution. After each change of the medium it was assured by repetitive scanning that the spectra were time-independent. All

1 A preliminary account of a similar study on the cytosolic isoenzyme from pig has recently been published by Metzler and colleagues (6).

2 The abbreviations used are: PEG, polyethylene glycol 6000; PEG solution, 20% (w/v) polyethylene glycol 6000/50 mM sodium phosphate (pH 7.5).

3 PEG increases the pH values of solutions buffered with sodium phosphate. Addition of 20% (w/v) PEG to a solution of 50 mM sodium phosphate increases pH values of 5.2, 6.0, 7.0, and 8.0 to 5.35, 6.25, 7.25, and 8.25, respectively. The pH values were determined with a glass electrode; the pH-increasing effect was also found using pH indicators.
RESULTS AND DISCUSSION

Microspectrophotometry of single crystals of aspartate aminotransferase (pyridoxal form) immersed in a PEG solution (see Footnote 3) gives an absorption spectrum ($\lambda_{max}$ 355 nm) which closely resembles that of the enzyme in solution (Fig. 1). In the region of protein absorbance, the ratio of $A_{300}$ to $A_{260} = 0.43$ of the crystal is almost the same as that of a solution of the enzyme (0.45). The ratio of the coenzyme absorbance at 355 nm to that of the coenzyme at 290 nm is 0.10, i.e. about 80% of that found for the holoenzyme in solution (0.13). On addition of cysteine sulfinate (0), the spectrum changes to that of the pyridoxamine form of the enzyme ($\lambda_{max}$ 333 nm). The conversion is complete (see inset to Fig. 1), indicating that both active sites of the crystalline enzyme interact with the substrate and undergo the half-reaction of transamination (see Footnote 4). Subsequent addition of oxalacetate results in the complete reverse half-reaction back to the pyridoxal form. In both the pyridoxal and the pyridoxamine form of the crystalline enzyme, the wavelengths of the absorbance maxima of the coenzyme at 355 nm and 333 nm, respectively, correspond to those of the enzyme in solution. The passage of an enzyme crystal through both half-reactions of transamination does not impair its crystalline order as judged by the quality of its x-ray diffraction pattern.

The intermediates of enzymatic transamination are readily observed after soaking the crystals in PEG solutions containing substrates or substrate analogs. Crystals of the pyridoxal form of the enzyme in PEG solutions containing 200 mM 2-oxoglutarate show an additional maximum at 430 nm about one-third the height of that at 355 nm (experiment not shown) as it has been found typical for the formation of an unproductive enzyme substrate adsorption complex in solution (10, 11). On addition of the substrate analog 2-methylaspartate (12), the crystals turn red, exhibiting an intense absorption band at 495 nm. The spectrum is very similar to that produced by interaction of 2-hydroxyaspartate with enzyme in solution (Fig. 2A). The absorption maximum at 495 nm has been assigned to the semiquinoid enzyme substrate intermediate.

1 In our triclinic crystals which contain one enzyme dimer per unit cell, the monomer has only two different orientations. Thus, in contrast to the behavior in solution, a random orientation distribution of the transition moments of the various chromophores of the enzyme, especially of the coenzyme, is expected. This situation results in anisotropy of the crystal absorption, the effect being wavelength-dependent. Therefore, identity of the value of the $A_{300}/A_{260}$ ratio as determined from measurements on our crystals with the value found for enzyme solutions would have been accidental. However, equally unlikely are orientations of the two crystallographically independent coenzyme chromophores in the enzyme dimer, such that the absorbance observed would be due to only one of them, the transition moment of the other being parallel to the direction of the incident light beam. There is no indication that coenzyme is lost during the crystallization procedure and the washing of the crystal (see legend of Fig. 1). The electron density maps of the native enzyme and the difference map (apo-holo enzyme) at 4.5 Å resolution indicate equal occupancy of both coenzyme binding sites (1).
containing 35 mM 2-methylaspartate, its spectrum was recorded after 7.1. (- - -) was recorded 5 min after the addition of 3-hydroxyaspartate. A, a crystal of the pyridoxal form of the enzyme was added to a PEG solution containing 20 mM 3-hydroxyaspartate/50 mM sodium phosphate (pH 7.1) (---) was recorded 5 min after the addition of 3-hydroxyaspartate. The same spectrum was obtained at pH 7.5. B, a crystal of the pyridoxal form of the enzyme was added to a PEG solution containing 35 mM 2-methylaspartate, its spectrum was recorded after 30 min (---). The same spectrum was obtained when 70 mM 2-methylaspartate was used and with enzyme that had been co-crystallized with 3-methylaspartate. The spectrum of the enzyme in solution (---) was taken in 35 mM 2-methylaspartate/50 mM sodium phosphate (pH 7.1). The same spectrum was obtained at pH 7.5.

FIG. 3 (right). Absorption spectrum of a crystal of aspartate aminotransferase at transamination equilibrium. The crystal was soaked in a PEG solution containing 0.2 mM oxalacetate and 4 mM aspartate for 30 min (---). At twice the concentrations of the substrates the same spectrum was obtained. For comparison, the spectrum of the enzyme in solution at the same substrate concentrations in 50 mM sodium phosphate at pH 7.5 is given (---). Lowering the pH of the solution to 7.1 did not change the spectrum.

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