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Microcrystals of Tryptophan Synthase $\alpha_2\beta_2$ Complex from *Salmonella typhimurium* Are Catalytically Active

Syed Ashrafuddin Ahmed,[‡,§] C. Craig Hyde,[∥] Gunter Thomas,[⊥] and Edith Wilson Miles*[,‡]

Laboratories of Biochemical Pharmacology and Molecular Biology, National Institute of Diabetes and Digestive and Kidney Diseases, and Laboratory of Ophthalmic Pathology, National Eye Institute, National Institutes of Health, Bethesda, Maryland 20892

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ABSTRACT: An improved and efficient method has been developed for the purification of the tryptophan synthase $\alpha_2\beta_2$ complex (EC 4.2.1.20) from *Salmonella typhimurium* containing a multicopy plasmid. Microcrystals prepared in 12% poly(ethylene glycol) 8000 containing 2.5 mM spermine are shown by scanning electron microscopy to have the same crystal habit as the larger crystals that are being used for structural analysis by X-ray crystallography. The average dimensions of the crystals are 33 μm (length) × 9 μm (width) × 3 μm (maximum thickness). Our finding that suspensions of microcrystals are active in several reactions catalyzed by the active sites of the α and β_2 subunits demonstrates that both active sites are functional in the crystal and accessible to substrates. Thus the larger crystals being used for X-ray crystallographic studies should form complexes with substrates and analogues at both active sites and should yield functionally relevant structural information. A comparison of the reaction rates of suspensions of microcrystals with those of the soluble enzyme shows that the maximum rate of the crystalline enzyme is 0.8 that of the soluble enzyme in the cleavage of indole-3-glycerol phosphate (α reaction), 0.3 that of the soluble enzyme in the synthesis of L-tryptophan by the β reaction or the coupled $\alpha\beta$ reaction, and 2.7 that of the soluble enzyme in the serine deaminase reaction. These small differences in rates probably reflect functional differences between the crystalline and soluble enzymes since the reaction rates of the microcrystals are calculated to be virtually free of diffusional limitation under these reaction conditions. The observed larger differences in the effects of ligands on the kinetic constants suggest that the transmission of ligand-induced conformational changes from one subunit to the other is reduced in the crystal due to crystal lattice forces.

The purpose of this work is to compare the functional properties of the crystalline $\alpha_2\beta_2$ complex of tryptophan synthase (EC 4.2.1.20) from *Salmonella typhimurium* with the properties of the enzyme in solution. The results are important in determining whether the structure of the enzyme which is being determined by X-ray crystallography (Ahmed et al., 1985)[1] is that of an active form of the enzyme, whether the crystalline enzyme can bind substrates and analogues, and whether the crystalline enzyme can undergo the same lig-

* Author to whom correspondence should be addressed.
‡ Laboratory of Biochemical Pharmacology.
§ Present address: Department of Biochemistry, The Bowman Gray School of Medicine, Winston-Salem, NC 27103.
∥ Laboratory of Molecular Biology.
⊥ Laboratory of Ophthalmic Pathology.

[1] A structural determination is in progress (C. C. Hyde, E. A. Padlan, S. A. Ahmed, E. W. Miles, and D. R. Davies). A preliminary report will be presented at the meeting of the American Society of Biological Chemists in Philadelphia, PA, June 8-11, 1987 (Hyde et al., 1987).

and-induced conformational changes as the soluble enzyme. Whereas some enzymes have identical reactions in the crystal and in solution, other crystalline enzymes are either inactive or differ from soluble enzymes in kinetic properties (Rupley, 1967). These differences are probably due to crystal lattice forces, which either distort the geometry of the active site or prevent ligand-induced conformational changes that occur in the soluble enzyme (Kasvinsky & Madsen, 1976; Alter et al., 1977; Makinen & Fink, 1977; Spilburg et al., 1977; Kirsten & Christen, 1983; Kirsten et al., 1983; Westbrook & Sigler, 1984; Ray, 1986).

Bacterial tryptophan synthase is an $\alpha_2\beta_2$ complex composed of two types of subunits, α and β_2, each of which has an active site and catalyzes a separate reaction, the α or β reaction, respectively [for reviews, see Miles (1979, 1986)]. The $\alpha_2\beta_2$ complex catalyzes both the α and β reactions and the $\alpha\beta$ reaction, which is the sum of the α and β reactions:

α reaction

indole-3-glycerol-P $\rightleftarrows$ indole + D-glyceraldehyde-3-P

β reaction

indole + L-serine $\rightarrow$ L-tryptophan + H_2O

$\alpha\beta$ reaction

indole-3-glycerol-P + L-serine $\rightarrow$ L-tryptophan + D-glyceraldehyde-3-P + H_2O

serine deaminase reaction

L-serine $\rightarrow$ pyruvate + NH_3

The β_2 subunit contains the cofactor pyridoxal phosphate and catalyzes the serine deaminase reaction as well as the β reaction and several other pyridoxal phosphate-dependent reactions (Kumagai & Miles, 1971; Ahmed et al., 1986). The serine deaminase activity of the $\alpha_2\beta_2$ complex is much lower than the activity of the β_2 subunit. Since ligands that bind to each subunit affect the steady-state kinetics of the reaction catalyzed by the complementary subunit in the $\alpha_2\beta_2$ complex, the $\alpha_2\beta_2$ complex appears to be regulated by ligand-induced conformational changes which are communicated between the α and β_2 subunits[2] (Creighton, 1970; Gschwin et al., 1979; Lane & Kirschner, 1983a,b).

In the present study we have developed a method for the rapid growth of microcrystals of the $\alpha_2\beta_2$ complex in 12% poly(ethylene glycol) 8000 containing 2.5 mM spermine by a modification of the method used for preparing large crystals suitable for X-ray crystallography (Ahmed et al., 1985). Scanning electron microscopy shows that the microcrystals have average dimensions of 33 μm (length) × 9 μm (width) × 3 μm (maximum thickness) and the same crystal habit as the large crystals. Steady-state kinetic data for four reactions collected for suspensions of microcrystals in 12% poly(ethylene glycol) 8000 containing 2.5 mM spermine are compared with the data for the soluble enzyme in 12% poly(ethylene glycol) 8000.

Experimental Procedures

Materials. Pyridoxal phosphate, poly(ethylene glycol) 8000, spermine tetrahydrochloride, and *N*,*N*-bis(2-hydroxyethyl)-glycine were obtained from Sigma Chemical Co. Indole-3-propanol phosphate was a generous gift from Dr. K. Kirschner (Biozentrum der Universität Basel, Switzerland). Indole-3-glycerol phosphate was prepared enzymatically (Hardman & Yanofsky, 1965).

Purification of the Tryptophan Synthase $\alpha_2\beta_2$ Complex. Step 1. Growth and Disruption of Cells and Ammonium Sulfate Fractionation. Salmonella typhimurium strain TB2211 containing multicopy plasmid pSTH8[3] was grown in a 50-L fermentor in a medium containing KH_2PO_4 (4.5 g/L), K_2HPO_4 (10.5 g/L), $(NH_4)_2SO_4$ (1.0 g/L), $MgSO_4$ (0.1 g/L), glucose (2.5 g/L), acid-hydrolyzed casein (1.0 g/L), L-tryptophan (10 mg/L),[4] and ampicillin (30 mg/L).[5] The harvested cells (145-g wet weight) were suspended in a final volume of 500 mL of buffer A [0.1 M potassium phosphate, pH 7.8, containing 10 mM β-mercaptoethanol, 0.02 mM pyridoxal phosphate, and 5 mM ethylenediaminetetraacetic acid (EDTA)] supplemented with 1 mM phenylmethanesulfonyl fluoride and disrupted by passage 2 times through a Gaulin homogenizer.[5] The homogenate (700 mL) was centrifuged at 12400*g* for 15 min at 5 °C, and the viscous, turbid supernatant was collected by rapid decantation. The supernatant (650 mL) was incubated at 25 °C for 2 h with stirring with 2 mL of 1 M $MgCl_2$, 5 mg of bovine pancreas deoxyribonuclease I (type IV, Sigma), and 2 mg of ribonuclease A (type III-A, Sigma) and then centrifuged at 30000*g* for 20 min at 5 °C. The turbid supernatant (620 mL, 2.8 × 10^6 units = 100% yield, 20 g of protein) collected by rapid decantation was treated with ammonium sulfate (109 g) with stirring at 5 °C, and the pH was adjusted to 7.8 with NH_4OH. After 20 min, the suspension was centrifuged for 20 min at 30000*g* at 5 °C, and the precipitate was discarded. Ammonium sulfate (73 g) was added to 575 mL of the supernatant at 5 °C. After 20 min, the suspension was centrifuged for 20 min at 30000*g* at 5 °C. The precipitate was dissolved in a minimum volume of buffer A and dialyzed against 5 L of buffer A for 16 h at 5 °C.

Step 2. Batchwise Treatment with DEAE-Sephacel. The dialyzed enzyme (100 mL, 1.5 × 10^6 units, 54% yield, 6.8 g of protein) was diluted to 250 mL with buffer A and applied to a bed of (diethylamino)ethyl- (DEAE-) Sephacel in buffer A that had been suction-filtered (without drying) onto a double layer of Whatman No. 4 filter paper disks (12.5 cm) in a Büchner funnel. The DEAE-Sephacel (400 mL) had been previously prepared by suspension followed by decantation or suction filtration in the following solutions for the times indicated: 1.5 L of 0.1 N NaOH (2 h); 2 L of water (6 times); 500 mL of 1 M potassium phosphate, pH 7.8 (1–7 days); 1 L of water (4 times); and 1 L of buffer A (2 times). The enzyme was filtered by gravity through the bed of DEAE-Sephacel. The bed was then washed first with 1.5 L of buffer A under mild suction and then with 1.6 L of buffer A in which the concentration of phosphate was increased to 0.2 M. The entire filtration procedure took about 1 h and yielded 1.2 × 10^6 units (43% yield, 2.3 g of protein) in the 0.2 M buffer wash. The presence of 10% of the initial activity (0.3 × 10^6 unit) in the 0.1 M buffer wash in this preparation, but not in others, indicates that the DEAE-Sephacel was overloaded in this preparation.

Step 3. Concentration and Crystallization by Ammonium Sulfate. The enzyme was precipitated by addition of ammonium sulfate (313 g added per liter of solution), collected by centrifugation for 20 min at 17700*g* at 5 °C, and dialyzed against 2 L of buffer A. This step was repeated if necessary to obtain 20 mL of enzyme at 77 mg/mL, which was then dialyzed for 16 h at 5 °C against 2 L of buffer A supplemented

[2] K. Kirschner, personal communication.

[3] We thank Dr. R. Bauerle for a gift of this strain.

[4] Subsequent studies show that a lower concentration of L-tryptophan (5 mg/L) gives a 2-fold higher initial specific activity of enzyme.

[5] We thank Dr. J. Shiloach for growth and disruption of bacteria.

with 170 g of ammonium sulfate added per 2 L. The suspension of crystals was incubated in a centrifuge tube for 2 h at 25 °C and centrifuged at 1600*g* for 15 min at 25 °C. The turbid supernatant was discarded. The precipitate of crystals was resuspended in 18 mL of buffer A supplemented with 1.53 g of ammonium sulfate and immediately centrifuged for 15 min at 1500*g*. The precipitate of crystals was resuspended in 18 mL of buffer A supplemented with 2.05 g of ammonium sulfate and centrifuged as above; this process was repeated once more. The final precipitate was resuspended in 10 mL of buffer B [50 mM sodium *N*,*N*-bis(2-hydroxyethyl)glycine, pH 7.8, containing 1 mM EDTA, 10 mM 2-mercaptoethanol, and 20 μM pyridoxal phosphate], dialyzed against 2 L of buffer B at 5 °C for 16 h, and centrifuged at 39000*g* for 15 min at 5 °C. The supernatant (23 mL) contained 1.1×10^6 units = 40% yield and had a specific activity of 1200 units/mg in the β reaction.

Preparation of Microcrystals. Microcrystals were prepared by a modification of the method used for the preparation of large crystals for X-ray crystallography (Ahmed et al., 1985). A solution (0.6 mL) of $\alpha_2\beta_2$ complex prepared above (10 mg/mL) in buffer B was added with a Pasteur pipet and with rapid mixing (5–10 s) to a second solution of 0.6 mL of buffer B supplemented with 12% poly(ethylene glycol) 8000 and 5 mM spermine that had previously been placed in a 1-mL syringe attached to a 0.45-μm nylon syringe filter (Schleicher and Schuell). The mixed solution was immediately filtered, and the filtrate, which was collected in a 1.5-mL Eppendorf tube, was mixed by inversion 3 times every 5 min for 30 min and then allowed to settle for 1 h at 25 °C. The clear supernatant solution was removed with a Pasteur pipet and discarded. The precipitated crystals were suspended in 0.5 mL of buffer A containing 6% poly(ethylene glycol) 8000 and 2.5 mM spermine and were transferred to a glass test tube (10 mm × 75 mm) that was then filled with the same buffer. The crystals were mixed thoroughly by inversion and were allowed to settle for 1.5 h. The supernatant solution containing some smaller crystals was removed and discarded. The remaining crystals in about 0.15 mL were resuspended in buffer B supplemented with 8% poly(ethylene glycol) 8000 and 2.5 mM spermine from which 2-mercaptoethanol was omitted, mixed by inversion, sedimented for 3 h as above, and resuspended in buffer B supplemented with 12% poly(ethylene glycol) 8000 and 2.5 mM spermine from which 2-mercaptoethanol was omitted. The sedimentation and resuspension in this buffer were again repeated after 6.5 and 12 h.

Scanning Electron Microscopy.[6] Microcrystals sedimented from buffer B supplemented with 12% poly(ethylene glycol) 8000 and 2.5 mM spermine (see above) were resuspended in buffer B supplemented with 10 mM spermine and allowed to settle for 3 h. The supernatant was carefully removed, and the crystals were resuspended in 100 μL of absolute ethanol and centrifuged 2 min at 5000*g* in a Beckman Airfuge. After repetition of this washing, 90 μL of the supernatant was discarded, the crystals were resuspended in 10 μL of ethanol, and 3 μL of the suspension was applied to a 3-mm × 3-mm segment of 0.45-μm Millipore filter mounted on colloidal silver paste (Ted Pella, Inc., Catalog No. 16032) on a sample-support stub. In method 1, the sample was air-dried for 10 min, placed into a sputter-coater (Denton Vacuum, Inc., Desk-1), and coated with an approximately 150-Å-thick layer of gold–palladium in argon atmosphere at 0.07 mmHg. Crystals were examined at magnifications ranging from 200× to 2000× and photographed at 15-kV accelerating voltage with a JEOL JSM-35 scanning electron microscope using polaroid P/N Type 55 film.

In method 2, 3 μL of the suspension of crystals was applied to a 3-mm × 3-mm segment of a 0.45-μm Millipore filter and immediately (in the presence of 1 mL of absolute ethanol) placed into the high-pressure chamber of a Samdri-780 critical point drying apparatus (Tousimis Research Corp.), using liquid CO_2 and the procedure of Anderson (1951). The sample was mounted, sputter-coated, and examined as above.

Assays of Activities of Crystalline and Soluble Enzymes. The four activities of the crystalline and soluble tryptophan synthase $\alpha_2\beta_2$ complexes were determined by spectrophotometric assays at 37 °C using 1-cm path length cells in a Cary 118 spectrophotometer. Standard assay procedures for the α and αβ reactions (Creighton & Yanofsky, 1970), the β reaction (Higgins et al., 1979; Miles et al., 1987), and the serine deaminase reaction (Crawford & Ito, 1964) were modified as indicated. All reaction mixtures contained 50 mM sodium *N*,*N*-bis(2-hydroxyethyl)glycine buffer, pH 7.8 (instead of the usual buffer), 50 μM pyridoxal phosphate, 1 mM EDTA, and 12% poly(ethylene glycol) 8000. NaCl or NH_4Cl was omitted. Assays of crystals also contained 2.5 mM spermine.

Assays of the crystalline tryptophan synthase were initiated by addition of 5–20 μL (10–30 μg) of the suspension of crystals. The initial rates in the β reaction were linear over the time period used (2–10 min) and were proportional to enzyme concentration (15–150 μg). Our finding that the rates were not changed by occasional stirring indicates that the crystals remained in suspension during the assay period. Since the rate of the crystalline enzyme is lower than the rate of the soluble enzyme in the β reaction (see Results), the linearity of the rate is evidence that the crystals are stable under the assay conditions and do not dissolve. In the absence of spermine, a progressive increase in the reaction rate occurs, which is due to dissoluton of the crystals. Further evidence that the microcrystals do not dissolve during assay of any of the four activities was obtained by filtering each assay mixture through a 0.45-μm filter. The filtrate contained less than 1% residual activity. Crystals recovered after assay in each of the reactions and examined by scanning electron microscopy (see above) showed no change in size or shape.

Assays of the soluble tryptophan synthase included 12% poly(ethylene glycol) 8000. Spermine was omitted since crystallization of the enzyme was initiated in its presence. However, spermine does not appear to affect the activity of either the soluble or crystalline enzyme since the soluble enzyme had the same initial rates in the α, β, and αβ reactions in the presence or absence of 2.5 mM spermine, in the absence of poly(ethylene glycol) 8000, or in the presence of 12% poly(ethylene glycol) 8000. Likewise, the crystalline enzyme had the same initial rate in the presence or absence of 2.5 mM spermine in the presence of 12% poly(ethylene glycol) 8000.

Protein Determination. The concentration of protein in the microcrystals in suspension and in the first two steps of purification was determined by the Bio-Rad dye binding protein assay (Bradford, 1976) using the soluble $\alpha_2\beta_2$ complex as a standard in the range 0.05–0.4 mg/mL. The crystals dissolve under the conditions of this assay. The concentration of purified $\alpha_2\beta_2$ complex was determined from the specific absorbance of $A_{278}^{1\%} = 6.0$ (Adachi et al., 1974).

Results and Discussion

Purification of the $\alpha_2\beta_2$ Complex of Tryptophan Synthase from Salmonella typhimurium. The new purification pro-

[6] We thank Dr. T. Kuwabara, Laboratory of Ophthalmic Pathology, National Eye Institute, for use of the facilities in his laboratory.

cedure described under Experimental Procedures is an improved and simplified version of the method used for the $\alpha_2\beta_2$ complex from *Escherichia coli* and from *Salmonella typhimurium* (Higgins et al., 1979; Miles et al., 1987). Whereas the *S. typhimurium* strain TB1533 (Δ*trp* LEDC1682 *trpR* 782) first used for purification of the enzyme for protein-folding studies (Yutani et al., 1984) and for crystallographic studies (Ahmed et al., 1985) contained the *trpA* and *trpB* genes in the genomic DNA, the strain used in this work lacks these genes in the genomic DNA but carries them on a multicopy plasmid derived from pBR322.[3] The amount of $\alpha_2\beta_2$ complex produced is estimated from the total units in the crude extract to be 11% of the total protein. This is about 2.6 times the amount obtained with the strain used previously. Subsequent studies have shown that if the concentration of L-tryptophan in the growth medium is reduced to 5 mg/L, the amount of enzyme in the crude extract is 20–25%.

The first step of the purification is improved by addition of deoxyribonuclease and ribonuclease, which degrade some of the viscous nucleic acid and allow better ammonium sulfate fractionation. Replacement of column chromatography on DEAE-Sephadex A50 by batchwise filtration through DEAE-Sephacel reduces the time for this step from 1–3 days to 1 h. The new crystallization step eliminates the second column chromatography step on Sepharose 4B. This step takes advantage of the unusual solubility properties of the $\alpha_2\beta_2$ complex from *S. typhimurium* (Miles et al., 1987). The enzyme exhibits two solubility minima in solutions of ammonium sulfate that are most pronounced at 24 °C; the precipitate in the first minimum at lower concentrations of salt is crystalline, whereas the precipitate at higher concentrations of salt is amorphous. Since all of the contaminating proteins remain in solution during the final crystallization step at low concentrations of ammonium sulfate, the procedure is an effective final purification step and yields enzyme that has two bands on sodium dodecyl sulfate gel electrophoresis. Although the overall yields (40%) are lower than the 60% previously reported by Miles et al. (1987), most of the losses occur during the first ammonium sulfate precipitation and result from the high concentration of enzyme in this preparation [4500 units/(mL of crude extract)]. No loss of activity occurred in this step in other preparations with lower initial concentrations of enzyme [1000–2000 units/(mL of crude extract)]. The use of an improved source of enzyme and the short time required for the purification (3 days) make this an efficient method for preparing both the native $\alpha_2\beta_2$ complex from *S. typhimurium* (this work) and mutant proteins prepared by site-specific mutagenesis.[7] Since the $\alpha_2\beta_2$ complex from *E. coli* does not crystallize at low concentrations of ammonium sulfate (Miles et al., 1987), the method cannot be used for purifying the *E. coli* enzyme.

Preparation and Scanning Electron Microscopy of Microcrystals. While growing crystals of the $\alpha_2\beta_2$ complex for X-ray crystallographic analysis using vapor diffusion from solutions containing poly(ethylene glycol) 8000 and spermine (Ahmed et al., 1985), we observed that the rate of crystallization and the size of crystals obtained were strongly influenced by the concentration of spermine. We have developed a method for preparing microcrystals (see Experimental Procedures) in which the enzyme is rapidly mixed with an equal volume of buffer containing 12% poly(ethylene glycol) 8000 and 5 mM spermine, immediately filtered to remove any amorphous materials formed, and allowed to crystallize. Sedimentation

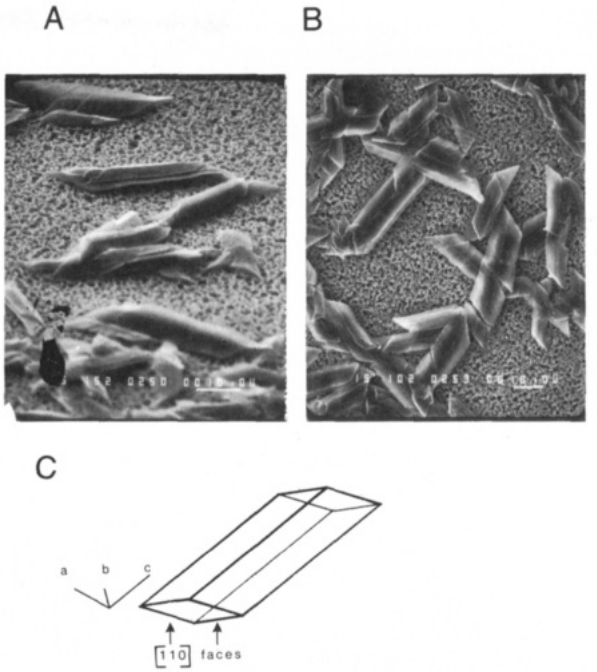

FIGURE 1: Microcrystals of the tryptophan synthase $\alpha_2\beta_2$ complex. Scanning electron micrographs of microcrystals coated with gold–palladium (A and B; see Experimental Procedures). The bar in each photograph represents 10 μm. The crystals in (A) are tilted and in (B) are flat. Diagram C shows form 1 of the crystals with [110] faces where *a* is the total width of the crystal, *b* is the maximum thickness, and $b/a = 0.34$. The average dimensions of the crystals in (B) are 9 μm wide × 33 μm long; the maximum thickness is calculated to be 3 μm, and the mean thickness is 1.5 μm (see text). The range of widths of 12 crystals is 6.7–13 μm.

under gravity of the crystals several times through a series of increasing concentrations of poly(ethylene glycol) 8000 stabilizes the crystals and selects crystals that appear fairly uniform in size and shape in scanning electron micrographs (Figure 1). Since these crystals appear to have the same habit as the large crystals used for X-ray crystallograhic analysis, the functional properties of the microcrystals and of the large crystals should be closely related except that the rates of the larger crystals are limited by the rate of diffusion of substrates.

In order to determine whether the microcrystals are free from this diffusional limitation, it is necessary to determine the average thickness, which is used in calculation of the enzymatic efficiency in the crystal (see below). The larger monoclinic crystals in space group *C*2 used for X-ray crystallography exist in two forms.[1] For form 1 with well-developed [110] faces, which is illustrated by a diagram in Figure 1C, the ratio of the maximum thickness to the total width of the face is 0.34. This ratio for the crystals of form 2 with [210] faces is 0.68. We assume that the microcrystals are form 1, since the tilted crystals in Figure 1A resemble the model in Figure 1C, both in the angled ridge traversing the length of each crystal and in the diamond-shaped end view seen in some crystals in Figure 1A. Furthermore, 9 out of 10 crystals being used for microspectrophotometric studies are in form 1.[8] Since the average width of the face of top views of the crystals (Figure 1B and other photographs not shown) is 9 μm, the maximum thickness is 9 μm × 0.34 = 3.0 μm. The mean thickness is then 3.0 μm divided by 2 = 1.5 μm.

Catalytic Activities of Crystalline and Soluble Tryptophan Synthase $\alpha_2\beta_2$ Complex. We have found that four activities of crystalline tryptophan synthase can be determined by

[7] H. Kawasaki, R. Bauerle, G. Zon, S. A. Ahmed, and E. W. Miles, work in progress, and Kawasaki et al. (1987).

[8] A. Mozzarelli, personal communication.

Table I: Steady-State Kinetic Constants of Crystalline and Soluble Tryptophan Synthase $\alpha_2\beta_2$ Complex from *Salmonella typhimurium*[a]

reaction	constant	soluble enzyme	crystalline enzyme	crystal/soluble
β	k_{cat} (s^{-1})	5.4	1.7	0.3
	$K_m'^{(indole)}$ (mM)	0.06	0.4	7
	$K_m'^{(serine)}$ (mM)	0.6	0.24	0.4
	$k_{cat}/K_m'^{(indole)}$ (s^{-1} mM^{-1})	90	4	0.05
	$k_{cat}/K_m'^{(serine)}$ (s^{-1} mM^{-1})	9	7	0.8
serine → pyruvate	k_{cat} (s^{-1})	0.06	0.16	3
	K_m^{serine} (mM)	0.02	0.06	3
	k_{cat}/K_m (s^{-1} mM^{-1})	3	2.7	0.9
α	k_{cat} (s^{-1})	0.3	0.2	0.8
	K_m^{IGP} (mM)	0.04	0.3	8
	k_{cat}/K_m^{IGP} (s^{-1} mM^{-1})	7	0.7	0.1
	K_I^{IPP} (mM)	0.004	0.04	10
$\alpha\beta$	k_{cat} (s^{-1})	3.7	1.0	0.3
	$K_m'^{(IGP)}$ (mM)	0.01	0.65	65
	$k_{cat}/K_m'^{(IGP)}$ (s^{-1} mM^{-1})	370	1.6	0.004
	K_I^{IPP} (mM)	0.0003	0.04	130

[a] All reactions were measured by spectrophotometric assays at 37 °C in 50 mM sodium *N,N*-bis(2-hydroxyethyl)glycine buffer, pH 7.8, containing 50 μM pyridoxal phosphate and 12% poly(ethylene glycol) 8000 as described under Experimental Procedures. Assay mixtures for suspensions of microcrystals also contained 2.5 mM spermine. Abbreviations: IGP, indole-3-glycerol phosphate; IPP, indole-3-propanol phosphate; K_m', apparent K_m in two-substrate reactions where one substrate is subsaturating.

spectrophotometric assays under conditions which prevent any solubilization of the crystals [12% poly(ethylene glycol) 8000 and 2.5 mM spermine (see Experimental Procedures)]. The soluble enzyme has been assayed in the presence of poly(ethylene glycol) 8000 but in the absence of spermine to prevent its crystallization. Neither spermine nor poly(ethylene glycol) 8000 appears to have significant effects upon the α or β activities of the crystalline or soluble enzyme.

We have determined the effect of substrate concentration on the rate of four reactions of the crystalline and the soluble tryptophan synthase $\alpha_2\beta_2$ complex (Table I). Since the experimental data are linear upon double-reciprocal transform in each case (data not shown), we conclude that the crystalline $\alpha_2\beta_2$ complex has only one population of active site and thus differs from aspartate aminotransferase, which displays lattice-induced functional asymmetry of the two subunits (Kirsten et al., 1983). This is consistent with the known space-group symmetry of the larger crystals, since the asymmetric unit is an $\alpha\beta$ pair (Ahmed et al., 1985).

A comparison of the maximum velocity of the crystalline $\alpha_2\beta_2$ complex with that of the soluble $\alpha_2\beta_2$ complex in each of the four reactions shows a complex relationship. Whereas the maximum velocities in the α reaction are similar with the two forms of the enzyme, the maximum velocity of the crystalline enzyme is 0.3 times that of the soluble enzyme in the β and $\alpha\beta$ reactions. In the latter two reactions, indole is added to L-serine at the active site of the β subunit to produce L-tryptophan. In contrast, the very slow serine deaminase reaction, which also occurs at the active site of the β subunit but which does not involve the β addition of indole, is about 3 times faster with the crystals than with the soluble enzyme. This increased serine deaminase activity might result if the Schiff base of amino acrylic acid were in a more hydrophilic environment in the crystal than in the soluble enzyme and thus more susceptible to hydrolysis to pyruvate and ammonia. There is evidence that the synthesis of L-tryptophan follows a branched mechanism involving isomeric enzyme–L-serine complexes and enzyme–L-tryptophan complexes (Lane & Kirschner, 1983b). Preliminary microspectrophotometric studies on single crystals of tryptophan synthase suggest that the equilibrium distribution of enzyme–L-serine complexes in the crystal is different from that in the soluble enzyme.[8]

The crystalline and soluble enzymes also differ markedly in K_m and apparent K_m (K_m') values for various substrates. Relatively small differences of the K_m for L-serine in the β reaction and the serine deaminase reaction were observed. The values of K_m' for indole and of K_m for indole-3-glycerol phosphate are about 8 times greater for the crystalline enzyme than for the soluble enzyme in the β and α reactions, respectively. The 65-fold difference between the K_m' for indole-3-glycerol phosphate in the $\alpha\beta$ reaction for the crystalline enzyme relative to that of the soluble enzyme probably results from different effects of L-serine on the binding and rate of cleavage of indole-3-glycerol phosphate at the active site of the α subunit in the crystalline and soluble $\alpha_2\beta_2$ complexes. Although addition of L-serine to the soluble enzyme increases the maximum rate of cleavage of indole-3-glycerol phosphate 13-fold and decreases the K_m' for indole-3-glycerol phosphate 4-fold, the effects on the crystalline enzyme are much smaller. These effects of L-serine on the soluble enzyme, first noted by Creighton (1970), have been attributed to a ligand-induced conformational change in the β subunit that is transmitted to the α subunit in the $\alpha_2\beta_2$ complex.[2]

Similar effects of L-serine upon the K_I of indole-3-propanol phosphate, an inhibitory analogue of indole-3-glycerol phosphate, are observed with the soluble enzyme but not with the crystalline enzyme (Table I) and have been previously reported for the soluble enzyme from *E. coli* (Gschwind et al., 1979; Lane & Kirschner, 1983a,b). The absence of an effect of L-serine on the binding of the substrate and analogue of the α subunit in the crystalline $\alpha_2\beta_2$ complex suggests that lattice forces in the crystal may prevent the transmission of a ligand-induced conformational change from the β subunit to the α subunit in crystals of the $\alpha_2\beta_2$ complex.

The reciprocal transmission of a ligand-induced conformational change from the α subunit to the β_2 subunit has been proposed[2] to explain the noncompetitive inhibition of the β reaction with respect to indole by indole-3-propanol phosphate (Kirschner & Wiskocil, 1970). It is difficult to compare this effect of indole-3-propanol phosphate upon the catalysis of the β reaction by the crystalline and soluble enzymes, since the K_I and K_m values for indole-3-propanol phosphate and indole are higher for the crystalline enzyme than for the soluble enzyme. We find the indole-3-propanol phosphate at a concentration of 0.1 mM (which is 2.5 times the K_I for the crystalline enzyme) results in 83% inhibition of the β activity of the soluble enzyme but only 51% inhibition of the β activity of the crystalline enzyme (both determined at 0.2 mM indole and 0.04 mM L-serine) (data not shown). These results suggest that the transmission of a ligand-induced conformational change from the α subunit to the β subunit is also reduced by lattice forces in the crystal.

Estimation of Diffusional Rate Limitation in Microcrystals.[9] Our aim has been to study the kinetics of crystalline tryptophan synthase $\alpha_2\beta_2$ complex under conditions not limited by the rate of diffusion of substrates into the crystals. Diffusional limitation depends on several factors that must be considered for each crystalline enzyme and reaction. These factors include the thickness of the crystal, the diffusion

[9] We thank Dr. R. C. Chatelier for his help in these estimations.

Table II: Comparison of Calculated and Observed Enzymatic Efficiencies in Microcrystals of Tryptophan Synthase[a]

reaction	substrate	K_m (mM)	$[S_0]$ (mM)	k_{cat} (s^{-1})	d_c (μm)	E_{calcd}[b] (%)	E'_{calcd}[c] (%)	E_{obsd}[d] (%)
β	indole	0.06	0.25	5.4	1.2	90	68	30
	L-serine	0.6	0.4	5.4	2.2	96	87	
serine deaminase	L-serine	0.02	0.2	0.06	9.7	100	99	300
α	indole-3-glycerol phosphate	0.04	0.5	0.28	7.0	100	99	80
$\alpha\beta$	indole-3-glycerol phosphate	0.01	0.5	3.7	1.9	95	83	30

[a] An estimate of d_c, the critical thickness of crystals that should allow 92% efficient catalysis of a given reaction, is calculated (Hoogenstraaten & Sluyterman, 1969) for each reaction:

$$d_c = [(K_m + [S_0])D'/k_{cat}[E]]^{1/2} \quad (1)$$

where K_m and k_{cat} are the steady-state kinetic constants for the soluble enzyme, [S] is the highest initial substrate concentration used under steady-state conditions for the crystalline enzyme, [E] is the millimolar concentration of the enzyme inside the crystal, and D' is the diffusion coefficient of substrate inside the crystal. D' was calculated by (Renkin, 1954)

$$D'/D = 1 - (a^2/r) \quad (2)$$

where D is the diffusion constant in water, a is the solute radius, and r is the average pore radius in the crystal. E, the estimated theoretical percent enzymatic efficiency in each reaction for crystals with an average thickness of d, is calculated by (Hoogstraaten & Sluyterman, 1969; Laidler & Bunting, 1980)

$$E = [\tanh \tfrac{1}{2}(d/d_c)/\tfrac{1}{2}(d/d_c)] \times 100 \quad (3)$$

We have used the following values in these calculations: K_m, K_{cat}, and $[S_0]$ are taken from Table I; [E] = 8.55 mM is calculated from the published data (Ahmed et al., 1985) which showed that there are 4 $\alpha\beta$ protomers = 4 asymmetric units per unit cell of 770×10^3 Å^3; r = 10 Å; a = 4 Å; and $D = 6 \times 10^{-6}$ cm^2/s. The value of D' calculated from these values of r, a, and D is 2.2×10^{-6} cm^2/s. D' is the most uncertain value since its calculation depends on several other estimated values. We have chosen a = 4 Å for all three substrates (M_r 105–287) since it is the average of the molecular radii calculated and used (3.6–4.4) for substances with molecular weights in this range (M_r 180–342) (Renken, 1954). The value of $D = 6 \times 10^{-6}$ cm^2/s is the average value estimated by Renkin (1954) and tabulated by Polson (1967) for substances in this molecular weight range. The value r = 10 Å is estimated from crystallographic data on larger crystals of tryptophan synthase.[1] These crystals contain channels up to 50 Å in diameter; the channels around the presumed active sites of the α and β_2 subunits are 15–20 Å wide and are easily accessible to outside solvent.[1] Although the value of $D' = 2.2 \times 10^{-6}$ cm^2/s is higher than that calculated by Hoogenstraaten and Sluytermann (1969) for other systems [$(0.2–0.6) \times 10^{-6}$ cm^2/s^{-1}], it is closer to that of Ray (1986) (1×10^{-6} cm^2/s) and of Spilburg et al. (1977) (3×10^{-6} cm^2/s^{-1}). [b] Calculated percent enzymatic efficiency for crystals with the observed mean thickness of 1.5 μm. [c] Calculated percent enzymatic efficiency for crystals with the observed maximum thickness of 3 μm. [d] Observed percent enzymatic efficiency (K_{cat}) of crystals relative to that of soluble enzyme (data taken from Table I).

coefficient of the substrate inside the crystal, the substrate concentration, the maximum rates, and the apparent K_m values for the substrates in each reaction examined (Hoogenstraaten & Sluytermann, 1969; Sluyterman & De Graaf, 1969).

Table II lists the factors which have been used to calculate the theoretical efficiency, E_{calcd}, of crystals in each of the reactions studied; footnote *a* in the table explains the equations and assumptions that have been used in these calculations. Since the theoretical efficiency, E_{calcd}, for crystals with a mean thickness of 1.5 μm is greater than 90% in each of the reactions, the rates of these reactions do not appear to be significantly limited by the rate of diffusion of substrates into the microcrystals. However, each crystal has a variable thickness due to its diamond-shaped cross section (Figure 1C) with the greatest thickness being 3.0 μm. The theoretical efficiency, E_{calcd}', for the thickest part of the crystal is lower (68–83%) than E_{calcd} (90–95%) for the faster β and $\alpha\beta$ reactions. However, both sets of calculated efficiencies are significantly higher than the observed efficiency (E_{obsd} = 30%) for the β and $\alpha\beta$ reactions, suggesting that there may be real differences between the crystalline and soluble forms of the enzyme. However, in view of the many assumptions in the calculations, it is possible that there are some diffusional effects (Ray, 1986). The important conclusion is that the crystals are quite active.

Conclusions. Our findings that microcrystals of tryptophan synthase $\alpha_2\beta_2$ complex catalyze reactions which occur at both the α and β active sites demonstrate that both the active sites are functional in the crystal and accessible to substrates. Thus the structure of the enzyme, which is being deduced by X-ray crystallography of larger crystals of the same type,[1] is that of an active form of the enzyme. These results and our finding that microcrystals did not crack or dissolve or change in habit after reactions with substrates lead us to hope that larger crystals will form complexes with ligands at both the α and β active sites suitable for studies by X-ray crystallography.

Some differences observed between the kinetic properties of the crystalline and soluble enzymes may reflect small differences in the conformation and flexibility of the enzyme in the two forms. These differences include the 3-fold increase in the rate of the very slow serine deaminase activity in the crystals (Table I) and the increased K_m and K_I values for some substrates and analogues (Table I). The largest effects of crystallization are on kinetic properties and have been ascribed to the transmission of ligand-induced conformational changes from the β subunit to the α subunit. Our recent finding that a single mutation in the α subunit (Arg-179 → Leu) appears to block these ligand-induced conformational changes and that this mutation may be in a domain interaction site[7] suggests that a rearrangement of the two α domains may occur during the ligand-induced conformational change which has been proposed for the wild type, soluble $\alpha_2\beta_2$ complex. This rearrangement of domains may be prevented in the crystal by crystal-packing forces.

Registry No. IGP, 4220-97-7; IPP, 40716-80-1; indole, 120-72-9; serine, 56-45-1; serine deaminase, 9014-27-1; poly(ethylene glycol) 8000, 25322-68-3; spermine, 71-44-3; tryptophan synthase, 9014-52-2.

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Purification and Properties of the Glycoprotein Processing *N*-Acetylglucosaminyltransferase II from Plants†

Taduez Szumilo,‡ G. P. Kaushal, and Alan D. Elbein*
Department of Biochemistry, University of Texas Health Science Center, San Antonio, Texas 78284
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ABSTRACT: The presence of an *N*-acetylglucosaminyltransferase (GlcNAc-transferase) capable of adding a GlcNAc residue to $GlcNAcMan_3GlcNAc$ was demonstrated in mung bean seedlings. This enzyme was purified about 3400-fold by using (diethylaminoethyl)cellulose and phosphocellulose chromatographies and chromatography on Concanavalin A–Sepharose. The transferase was assayed by following the change in the migration of the [^{3}H]mannose-labeled GlcNAcβ1,2Manα1,3(Manα1,6)Manβ1,4GlcNAc on Bio-Gel P-4, or by incorporation of [^{3}H]GlcNAc from UDP-[^{3}H]GlcNAc into a neutral product, $(GlcNAc)_2Man_3GlcNAc$. Thus, the purified enzyme catalyzed the addition of a GlcNAc to that mannose linked in α1,6 linkage to the β-linked mannose. GlcNAcβ1,2Manα1,3(Manα1,6)Manβ1,4GlcNAc was an excellent acceptor while Manα1,6(Manα1,3)Manβ1,4GlcNAc, Manα1,6(Manα1,3)Manα1,6-(Manα1,3)Manβ1,4GlcNAc, and Manα1,6(Manα1,3)Manα1,6[GlcNAcManα1,3]Manβ1,4GlcNAc were not acceptors. Methylation analysis and enzymatic digestions showed that both terminal GlcNAc residues on $(GlcNAc)_2Man_3GlcNAc$ were attached to the mannoses in β1,2 linkages. The GlcNAc transferase had an almost absolute requirement for divalent cation, with Mn^{2+} being best at 2–3 mM. Mn^{2+} could not be replaced by Mg^{2+} or Ca^{2+}, but Cd^{2+} showed some activity. The enzyme was also markedly stimulated by the presence of detergent and showed optimum activity at 0.15% Triton X-100. The K_m for UDP-GlcNAc was found to be 18 μM and that for $GlcNAcMan_3GlcNAc$ about 16 μM.

The N-linked or asparagine-linked glycoproteins occur in plants as well as in animals (Kornfeld & Kornfeld, 1985; Lehle & Tanner, 1983), and the plant glycoproteins may contain either high-mannose or modified oligosaccharide chains (Takahashi et al., 1986). Some of these modified or complex oligosaccharides of plants have a galactose-β1,4GlcNAcβ1,2-sequence attached to each of the α-linked mannoses of the trimannose core, in close analogy to the complex structures of animal cells (Ishihara et al., 1979; Kobata, 1984). However,

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‡ Present address: Department of Biochemistry, Medical School, Lublin, Poland.

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