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Enzymological Evidence for Separate Pathways for Aflatoxin B_1 and B_2 Biosynthesis

Deepak Bhatnagar,*,‡ Thomas E. Cleveland,‡ and David G. I. Kingston§

Southern Regional Research Center, U.S. Department of Agriculture/Agricultural Research Service, P.O. Box 19687, 1100 Robert E. Lee Boulevard, New Orleans, Louisiana 70179, and Department of Chemistry, Virginia Polytechnic Institute and State University, Blacksburg, Virginia 24061

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ABSTRACT: Aflatoxins B_1 (AFB_1) and B_2 (AFB_2) are biologically active secondary metabolites of *Aspergillus flavus* and *Aspergillus parasiticus*. These toxins are synthesized by the fungi from pathway precursors: sterigmatocystin (ST) → *O*-methylsterigmatocystin (OMST) → AFB_1; dihydrosterigmatocystin (DHST) → dihydro-*O*-methylsterigmatocystin (DHOMST) → AFB_2. The late stages of AFB_1 synthesis are carried out by two enzyme activities, a methyltransferase (MT) (ST → OMST), and an oxidoreductase (OR) (OMST → AFB_1). Properties of the purified MT have been identified in a previous investigation [Bhatnagar et al. (1988) *Prep. Biochem. 18*, 321]. In the current study, the OR was partially purified (150-fold of specific activity) from fungal cell-free extracts and characterized with extended investigation of the late stages of AFB_1 and AFB_2 synthesis. Whole cells of an isolate of *A. flavus* (SRRC 141), which produce only AFB_2, were able to produce AFB_1 in ST and OMST feeding studies; the results suggested that the enzymes involved in AFB_2 biosynthesis also carry out AFB_1 synthesis. Substrate competition experiments carried out with the OR showed that an increasing concentration of either OMST or DHOMST in the presence of a fixed, nonsaturating concentration of either DHOMST or OMST, respectively, resulted in a decline in production of one aflatoxin (B_1 or B_2) with a corresponding increase in the synthesis of the other toxin (B_2 or B_1). OMST was a preferred substrate (K_m, 1.2 μM) for the oxidoreductase as compared to DHOMST (K_m, 13.4 μM). Similar, substrate competition experiments showed that ST (K_m, 2.0 μM) was a preferred substrate over DHST (K_m, 22.5 μM) for a homogeneous MT. The results suggest that AFB_1 and AFB_2 synthesis is catalyzed by common enzymes that use separate precursors as substrates for the synthesis of each toxin. A biosynthetic grid for the AFB_1 and AFB_2 synthesis is presented on the basis of enzyme substrate specificity studies and cellular conversions of pertinent metabolites.

Aflatoxins B_1 and B_2 (AFB_1 and AFB_2)[1] are toxic secondary metabolites produced by *Aspergillus flavus* and *Aspergillus parasiticus*. The mode of action, metabolism, and chemical biosynthesis of AFB_1 have been extensively studied (Malik, 1982; Bennett & Christensen, 1983; Townsend, 1986). Biotransformation experiments with mutants of *A. parasiticus* blocked in toxin formation and ^{14}C-radiolabeled precursor studies (Bhatnagar et al., 1987; McCormick et al., 1987) have resulted in the following scheme of precursors in aflatoxin B_1 biosynthesis: norsolorinic acid → averantin → averufanin → averufin → versiconal hemiacetal acetate → versicolorin A → sterigmatocystin (ST) → *O*-methylsterigmatocystin (OMST) → aflatoxin B_1 (Figure 1). Several analogues of aflatoxin B_1 and its precursors (Figure 2) have been identified from extracts of *Aspergillus* mycelia (Cole & Cox, 1981).

Fractionation of subcellular *A. parasiticus* mycelial extracts resolved two pertinent enzyme activities (Cleveland et al., 1987b): (1) a methyltransferase (MT) catalyzing the conversion of ST to OMST and (2) an oxidoreductase (OR) catalyzing the OMST to AFB_1 conversion (Figure 1). The methyltransferase has recently been purified to homogeneity in our laboratory (Bhatnager et al., 1988); this enzyme is the first catalyst specific for aflatoxin biosynthesis (Cleveland & Bhatnagar, 1989) that has been purified to homogeneity.

There have been many conflicting hypotheses concerning the biosynthetic pathways of AFB_2. Several reports (Maggon & Venkitasubramanian, 1973; Biollaz et al., 1970; Heathcote et al., 1976; Thomas, 1965) suggested that AFB_2 may be metabolically related to AFB_1 by a direct interconversion process, whereas others (Dutton et al., 1985; Floyd et al., 1987; Dutton, 1988) had postulated that AFB_1 and AFB_2 may be synthesized in fungal mycelia by separate pathways, the branch

* Corresponding author.
‡ Southern Regional Research Center, USDA/ARS.
§ Department of Chemistry, Virginia Polytechnic Institute and State University.

[1] Abbreviations: AFB_1, aflatoxin B_1; AFB_2, aflatoxin B_2; AFB_{2a}, aflatoxin B_{2a}; ST, sterigmatocystin; OMST, *O*-methylsterigmatocystin; DHST, dihydrosterigmatocystin; DHOMST, dihydro-*O*-methylsterigmatocystin; HST, sterigmatocystin hemiacetal; HOMST, *O*-methylsterigmatocystin hemiacetal; MST, 5-methoxysterigmatocystin (7-hydroxy-6,10-dimethoxydifuroxanthone); HMST, 5-methoxysterigmatocystin hemiacetal; EST, sterigmatocystin ethoxy acetal; MT, methyltransferase; OR, oxidoreductase.

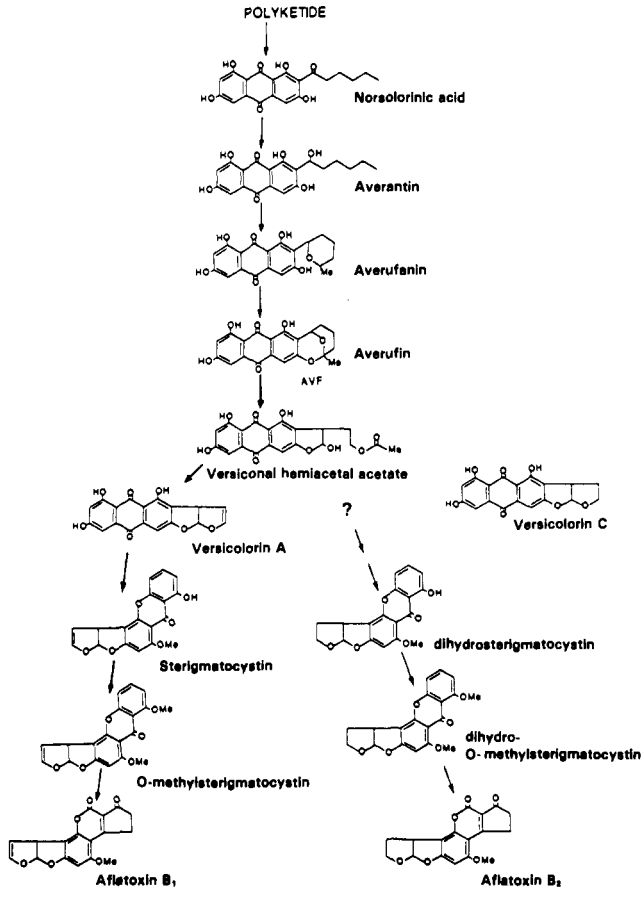

FIGURE 1: Currently accepted pathway of aflatoxin biosynthesis. The branch point in the synthesis of aflatoxins B_1 and B_2 has not yet been established, but versicolorin C is postulated to be an aflatoxin B_2 precursor.

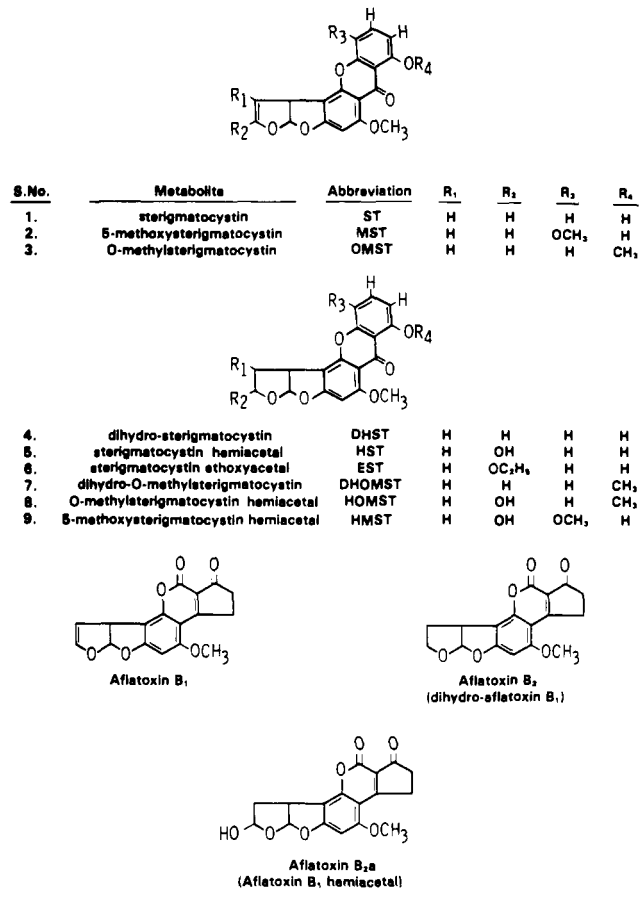

FIGURE 2: Structures of various aflatoxin and sterigmatocystin analogues.

arising at the metabolic precursor versiconal hemiacetal acetate (VHA) (Dutton et al., 1985; Maggon et al., 1977). It has also been proposed that versicolorin C (Dutton et al., 1985) and 5-hydroxydihydrosterigmatocystin (Elsworthy et al., 1970) may be AFB_2 precursors. Previous investigations also demonstrated that unique secondary metabolites, a hydroxylated analogue of OMST (OMST hemiacetal, HOMST), and a dihydro analogue of OMST (DHOMST) isolated from cultures of *A. parasiticus* (SRRC 2043) were converted to AFB_2, but not AFB_1, by cultures of *A. parasiticus* (SRRC 163) (Cleveland et al., 1987a; Cleveland, 1989); the results suggested divergent pathways for AFB_1 and AFB_2 synthesis (Figures 1 and 2). Another report (Yabe et al., 1988) also stated that DHOMST and a dihydro analogue of ST, DHST, are unique precursors in AFB_2 synthesis. Results of that study with cell-free extracts also suggested, on the basis of cofactor requirements of the enzymes (Cleveland & Bhatnagar, 1987), that common enzymes might be involved in ST conversion to OMST and AFB_1 and DHST conversion to DHOMST and AFB_2. The branch point in the synthesis of these toxins had not been experimentally established.

In the current study, a homogeneous preparation of the methyltransferase and a partially purified preparation of the oxidoreductase have been linked to the late stages of both the AFB_1 and AFB_2 biosyntheses. However, ST and OMST are the preferred substrates of the MT and OR, respectively, relative to DHST and DHOMST. A metabolic grid indicating an interrelationship between AFB_1 and AFB_2 precursors has also been presented, on the basis of the substrate specificity of the enzymes in the late stages of aflatoxin biosynthesis and the cellular interconversions of key pathway intermediates.

Materials and Methods

Strain and Growth Conditions. The *A. parasiticus* mutant strains used in this study were nonaflatoxigenic isolates designated AVN-1 (SRRC 163) and OMST-1 (SRRC 2043). AVN-1 accumulates averantin but can convert ST to OMST and OMST to AFB_1, whereas OMST-1 accumulates OMST even when the AFB_1 precursor ST is added to the culture medium (Bhatnagar et al., 1987). A strain of *A. flavus* (SRRC 141) that produces only AFB_2 (Schroeder & Carlton, 1973) without producing AFB_1 was also used in this study.

The fungi were grown on growth medium (GM) (Adye & Mateles, 1964) for 3–4 days. A 1.0-mL portion of a culture spore suspension (10^8 spores/mL) was transferred to 1 L of GM in 2.8-L Fernback flasks, and the flasks were incubated on a shaker incubator at 150 rpm and 28 °C. The mycelia were harvested from GM by vacuum filtration and washed extensively with sterile distilled water. For metabolite feeding studies, 1 g (wet weight) of 3–4-day-old mycelia was added to each 50-mL flask containing 20 mL of incubation medium (low-sugar replacement medium + 20 μL of metabolite solution in acetone) as described earlier by Bhatnagar et al. (1987). The contents were incubated for 20 h at 30 °C with constant shaking at 150 rpm.

Metabolites. ST and OMST were obtained from Sigma Chemical Co. (St. Louis, MO). 5-Methoxy-ST (MST) was obtained from Makor Biochemicals (Jerusalem, Israel). DHST and EST were prepared by the procedure of Chen et al. (1977), and DHOMST was extracted from SRRC 2043 cultures as described earlier (Cleveland, 1989). HST, HOMST, and HMST were synthesized from ST, OMST, and MST, respectively, by trifluoroacetic acid treatment as described earlier by Cleveland et al. (1987a). Identities of the

Table I: Purification Scheme for the Oxidoreductase from *A. parasiticus* (SRRC 163) Mycelia[a]

purification steps[b]	total vol (mL)	total protein (mg)	total enzyme act. (μmol of AFB_1/min)	sp act.	fold purification of sp act.
20000*g* supernatant	2200	6150	1456	0.23[c]	1[c]
CDR elution	200	930	1412	1.52	6.6
ammonium sulfate precipitation (36%–65% cut)	4	240	1303	5.43	23.6
QMA anion exchange	25	80	942	11.78	51.2
Bio-Gel P-200	15	6	206	34.11	148.3

[a] 500 g wet weight of starting material. [b] The purification steps are as described in the text. [c] Specific activity = μmol of AFB_1/(mg of protein·min).

metabolites were established by chromatographic determination on thin-layer chromatographic plates (TLC) and by mass spectrometry with a Finnigan 4000 spectrometer, by using analytical standards or by comparison with established spectra (Cole & Cox, 1981; Cleveland, 1989).

Extraction and Analysis of Metabolites. After the desired length of incubation in the various media, the mycelia were extracted by standard procedures (Bhatnagar et al., 1987). The metabolites were primarily separated by TLC with ether/methanol/water (96:3:1) (v/v/v) as the developing solvent. The mobilities of various metabolites in the solvent system were ST, 0.97; OMST, 0.44; AFB_1, 0.37; AFB_2, 0.35; HST, 0.96; HOMST, 0.25; DHST, 0.98; DHOMST, 0.31; AFB_{2a}, 0.28; MST, 0.95; HMST, 0.91; and EST, 0.87. Further purification of secondary metabolites was carried out by Sephadex LH-20 column chromatography (column size 3.5 × 15 cm) with methanol or acetone as the developing solvents (McCormick et al., 1988). The metabolites and aflatoxins were quantitated by comparison of the areas of peaks of fluorescent scans at 310 or 360 nm of spots on TLC plates against peaks of known standards run on the same plate, by using a Shimadzu dual-wavelength TLC scanner, CS-930 (Bhatnagar et al., 1987; Cleveland et al., 1987a). Quantities of metabolites were also determined spectrophotometrically (Shimadzu UV–visible dual-beam spectrophotometry, UV-160) by using established extinction coefficients (Bhatnagar et al., 1987; Cleveland et al., 1987a; Cleveland, 1989; Chen et al., 1977; Yabe et al., 1988; Cole & Cox, 1981).

Preparation of Cell-Free Mycelial Extracts. After 84 h of incubation, mycelial pellets were harvested by vacuum filtration and washed extensively with buffer A [0.05 M potassium phosphate, pH 7.5, 10% (v/v) glycerol, and 2 μM β-mercaptoethanol (Bhatnagar et al., 1988)], containing 100 μM phenylmethanesulfonyl fluoride. Mycelia were frozen with liquid nitrogen and pulverized to a fine powder under liquid nitrogen in a Waring blender; this procedure for cell disruption was found to be the most efficient method for recovery of enzyme activity (Bhatnagar et al., 1989). The powdered mycelia were suspended in buffer A. The resultant brei was filtered through one layer of Miracloth, and cellular debris was removed by centrifugation at 5000*g*. The supernatant fraction was further clarified by centrifugation at 20000*g*. The supernatant fraction from the 20000*g* centrifugation was used for the purification of the enzymes.

Enzyme Purification. All the purification steps using cell-free extracts of mycelia of *A. parasiticus* (SRRC 163) were carried out at 4 °C. The methyltransferase was purified to homogeneity according to the procedure developed in our laboratory (Bhatnagar et al., 1988). The chromatographic columns used for the partial purification of the oxidoreductase were prepared similarly to those used for MT purification (Bhatnagar et al., 1988). The OR purification was carried out from cell-free mycelial extracts by CDR (Cell Debris Remover, a cellulosic weak anion exchanger, Whatman) treatment, ammonium sulfate precipitation, QMA ACELL (Waters) ion exchange, and Fracto-Gel TSK HW-50F (EM Science) filtration chromatography.

Enzyme Assays. The enzyme activity (MT or OR) measurements were carried out at 29 °C. The reaction mixtures routinely consisted of a final volume of 1 mL of 50 mM phosphate buffer, pH 7.5, 10% glycerol, 2 mM β-mercaptoethanol, 500 μM *S*-adenosylmethionine (for MT) or 500 μM NADPH (for OR), and the enzyme fraction (usually 10–100 μL) in an unsealed glass vial (10 mL). The assays were initiated by the addition of metabolites such as ST (for MT) or OMST (for OR) in 10 μL of acetone to give a final concentration of 50 μM in the incubation medium. Concentrations of metabolite substrates or cofactors were also varied for K_m determinations and substrate competition studies. Reactions were carried out for between 10 min and 1 h with constant shaking (150 rpm) and terminated by addition of 4 mL of methylene chloride to the reaction mixtures followed by vigorous shaking in capped assay vials. The organic solvent phase of the reactions was removed and dried under nitrogen, and the dried residue was dissolved usually in 50–100 μL of acetone. The metabolites and/or aflatoxins produced in these reactions were analyzed as described earlier in this section. One unit of enzyme activity was expressed as micromoles of metabolite or aflatoxin produced per milligram of protein per minute.

All experimental values from enzyme activity determinations represent the means and standard errors of at least three independent determinations; linear regression analyses were used to determine the linearity of plots in the determination of K_m values from Lineweaver–Burk plots.

Protein Concentration. Total protein concentration was determined by the Coomassie blue G-250 dye-binding method of Sedmak and Grossburg (1977), with ovalbumin as a standard.

Gel Electrophoresis. The purity of protein preparations was determined by denaturing or nondenaturing polyacrylamide gel electrophoresis (Bhatnagar et al., 1988).

Results

Methyltransferase Purification. The methyltransferase purified for this study was nearly homogeneous and consisted of two subunits (110 kDa and 58 kDa), similar to earlier preparations (Bhatnagar et al., 1988). The specific activity of the enzyme was 1.95 μmol of OMST/(mg of protein·min).

Oxidoreductase Purification

Step 1. CDR (Cell Debris Remover) Treatment. Total OR activity in the mycelial extracts was 1456 units (Table I). CDR added to the cell-free extracts bound all the OR activity since none of the activity was recovered in the flow-through from the CDR column. The CDR column was eluted with 200 mL of 1.0 M KCl at a flow rate of 25 mL/h, and 100 μL of the effluent was assayed for OR activity; 86% of the OR

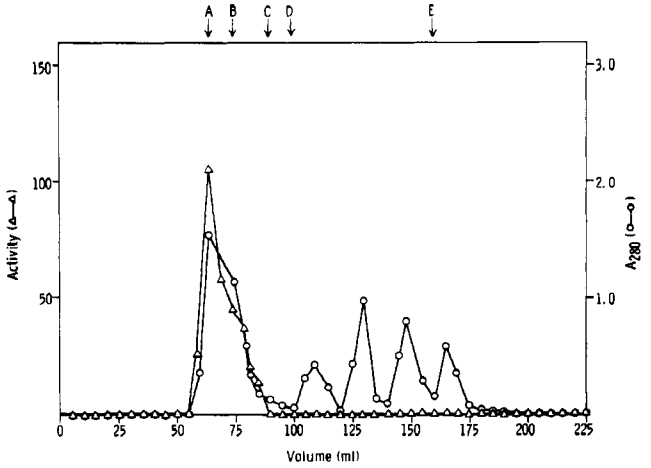

FIGURE 3: Fracto-Gel chromatography of QMA ion-exchange chromatographically purified oxidoreductase. The molecular masses of standards, designated as letters, are (A) blue dextran, 2000 kDa, (B) aldolase, 158 kDa, (C) bovine serum albumin, 68 kDa, (D) ovalbumin, 44 kDa, and (E) carbonic anhydrase, 30 kDa. Shown are A_{280} (O) and enzyme activity (Δ).

activity and about 15% of the total extracted protein were recovered in the effluent. The eluate was dialyzed extensively against buffer A.

Step 2. Ammonium Sulfate Precipitation. The proteins in the dialyzed eluate from the CDR column were fractionated by addition of solid ammonium sulfate in three steps between 0% and 35%, 36% and 65%, and 66% and 90% saturation with constant stirring for 1 h at 4 °C. The precipitated protein was removed by centrifugation at 30000*g* for 30 min. The protein in each case was resuspended in 4 mL of buffer A. The protein fractions were extensively dialyzed in buffer A and assayed for OR activity. The activity was found mainly in the 36%–65% ammonium sulfate precipitated proteins. This fraction contained nearly 25% of the total protein and 90% of the OR activity.

Step 3. QMA ACELL Chromatography. The protein material from the ammonium sulfate precipitation was diluted to 50 mL and applied to a QMA ACELL anion-exchange column (2.5 × 20 cm) previously equilibrated with buffer A at a flow rate of 50 mL/h. After elution of unbound protein (250 mL of buffer A), a linear gradient of KCl in buffer A with a slope of 0–0.5 M and a total volume of 250 mL was developed at a flow rate of 25 mL/h, and fractions of 5.5 mL were collected. Aliquots (100 μL) were assayed for OR activity; the peak OR activity eluted at 250 mM KCl. Fractions with pronounced oxidoreductase activity were pooled and concentrated to nearly 3 mL in an Amicon Model 8050 ultrafiltration cell with a PM-10 Diaflo membrane. The protein was exhaustively dialyzed in buffer A.

Step 4. Fracto-Gel TSK HW-50F Chromatography. The concentrated fraction from the previous step was chromatographed on a Fracto-Gel column. Fractions (5 mL) were collected and assayed for OR activity. The gel-filtration profile indicated that there were five distinct protein (A_{280}) peaks (Figure 3); the OR activity migrated with the first peak, one fraction removed from the void volume (<200 kDa), and migrated prior to aldolase (158 kDa). The fractions showing OR activity were pooled, concentrated to nearly 5 mL, and stored at −20 °C.

The purified OR (from the Fracto-Gel column) when analyzed by SDS–PAGE showed seven major protein bands at approximately 165, 105, 88, 64, 43, 38, and 26 kDa. The purified protein preparation migrated as four major protein bands on nondenaturing PAGE, indicating that this preparation was not a homogeneous preparation of the OR. None of the bands, when excised and extracted from an unstained gel, demonstrated OMST to AFB_1 conversion activity. The partially purified OR from the Fracto-Gel column was used for substrate specificity studies in the elaboration of the synthesis of AFB_1 and AFB_2.

Table II: Conversion of Sterigmatocystin (ST), Dihydrosterigmatocystin (DHST), Sterigmatocystin Hemiacetal (HST), and Aflatoxin B_{2a} (AFB_{2a}) by Intact Cells of *A. parasiticus* (SRRC 163) to Aflatoxins B_1 or B_2

added metabolite(s)	aflatoxins produced[a] (μg/g of mycelial wet weight)	
	AFB_1	AFB_2
none	ND[b]	ND
ST (10 μg)	3.8 ● 0.7	ND
DHST (10 μg)	ND	2.4 ± 0.3
HST (50 μg)	ND	2.5 ● 0.4
HOMST (50 μg)	ND	3.9 ● 0.5
ST (10 μg) + HST (50 μg)	3.4 ± 0.7	1.4 ± 0.3
DHST (10 μg) + HST (50 μg)	ND	4.0 ± 0.5
AFB_{2a} (100 μg)	ND	ND
AFB_{2a} (250 μg)	ND	ND
AFB_{2a} (400 μg)	ND	ND

[a] The aflatoxins were produced by SRRC 163 intact cells in a low-sugar replacement medium as described in the text. Values represent the means and standard errors of at least three separate experiments. [b] ND, none detected.

Properties of the Oxidoreductase. The enzyme activity was optimum at pH 7.5 and between 25 and 30 °C, properties similar to previous observations with cell-free extracts (Cleveland & Bhatnagar, 1987). OMST (K_m, 1.2 μM) and NADPH (K_m, 15 μM) were the substrates for the enzyme activity; the activity in the pooled fractions was 34 μmol of OMST/(mg of protein·min) at 50 μM OMST and 500 μM NADPH. The activity was linear from 50 to 250 μg of total protein up to 1 h when determined with 100 μg of total protein in the reaction mixtures.

Conversion of Metabolites to Aflatoxins B_1/B_2 by Whole Cells of A. parasiticus and A. flavus. Earlier studies demonstrated conversion of HOMST to AFB_2 (Cleveland et al., 1987a). To study the role of HST in the aflatoxin biosynthetic pathway, HST (50 μg) was fed to the *A. parasiticus* mutant AVN-1 (SRRC 163). HST was converted only to AFB_2, but only 20% as efficiently as the AFB_2 precursor DHST (Table II) and nearly 65% as efficiently as HOMST. When HST (250 μg) was fed to 3-day-old cultures of the *A. parasiticus* mutant OMST-1 (SRRC 2043), no increase in the accumulation of HOMST (15.8 μg/g dry weight of mycelia) was observed (data not shown). There was no degradation or interconversion of these metabolites when they were incubated with autoclaved (dead) cells of SRRC 163 or SRRC 2043 in the low-sugar resting medium. The results demonstrate that there is conversion of HST and HOMST to AFB_2 but not to AFB_1 and that there may be no direct conversion of HST to HOMST in intact fungal cells.

Incubation of AFB_{2a} for 6–24 h with 3-day-old cultures of SRRC 163 did not result in the production of any detectable AFB_2 in the extracted metabolites, and all the AFB_{2a} was recovered from the incubation medium (Table II).

When a strain of *Aspergillus flavus* (SRRC 141) that produces only AFB_2 on several natural substrates (Schroeder & Carlton, 1973) was fed ST, OMST, DHST, and DHOMST, or combinations of metabolites (Table III), ST and OMST were converted to AFB_1 without any increase in AFB_2 production, whereas DHST and DHOMST feeding increased AFB_2 production without AFB_1 synthesis. Both

Table III: Conversion of 10 μg of Aflatoxin Precursors to Aflatoxins B_1 (AFB_1) and B_2 (AFB_2) by Submerged Cultures of an AFB_2-Producing *A. flavus* (SRRC 141)

metabolite added[a]	aflatoxins produced[b] (μg/g of mycelial wet weight)	
	AFB_1	AFB_2
none	ND[c]	8.3 ± 1.0
ST	2.6 ± 0.4	6.8 ± 1.3
DHST	ND	9.9 ± 2.1
ST + DHST	1.8 ± 0.3	9.1 ± 1.8
OMST	4.1 ± 0.6	7.2 ± 1.6
DHOMST	ND	10.9 ± 2.4
OMST + DHOMST	3.1 ± 0.5	8.8 ± 2.1

[a] ST, sterigmatocystin; DHST, dihydrosterigmatocystin; OMST, *O*-methylsterigmatocystin; DHOMST, dihydro-*O*-methysterigmatocystin. [b] The aflatoxins were produced by SRRC 141 intact cells in a low-sugar replacement medium, as described under Materials and Methods. Values represent the mean and standard errors of at least three separate experiments. [c] ND, none detected.

Table IV: Kinetic Parameters for the Methyltransferase with Sterigmatocystin (ST) or Dihydrosterigmatocystin (DHST) as Substrate[a]

substrate	app K_m (μM)	V_{max} [μmol/(min·mg)]
ST	2.0	10.2 ± 2.6 (OMST)[b]
DHST	22.5	7.8 ± 1.4 (DHOMST)[b]

[a] K_m and V_{max} were determined by Lineweaver–Burk plots. Reactions were performed for 10 min as described under Materials and Methods by using a fixed, saturating concentration of *S*-adenosylmethionine (500 μM). The concentrations of ST and DHST were varied from 0.2 to 50 μM and 4 to 200 μM, respectively. [b] The product detected was OMST, *O*-methylsterigmatocystin, or DHOMST, dihydro-*O*-methylsterigmatocystin.

AFB_1 and AFB_2 syntheses were detected when ST in combination with DHST or OMST in combination with DHOMST were fed to the whole cells of SRRC 141. The substrate metabolites were recovered completely intact when incubated with autoclaved (dead) cells of SRRC 141 as a control. The results suggest that the fungal strain (SRRC 141) had the enzymes required for the conversion of ST to OMST and aflatoxin B_1; but there did not appear to be any interrelationship between aflatoxin B_1 and aflatoxin B_2 precursors, namely ST/OMST and DMST/DHOMST, respectively. It was, therefore, postulated that the same enzymes may be catalyzing the two reactions (AFB_1 and AFB_2 syntheses) from the two sets of substrates. Substrate specificity studies were carried out to determine if the MT and OR involved in AFB_1 biosynthesis utilized the AFB_2 precursors.

Substrate Specificity Studies with the Enzymes in the Final Steps of Aflatoxin Biosynthesis. Kinetic studies with the methyltransferase using ST or DHST as a substrate demonstrated that both ST and DHST are equally good substrates for this enzyme; but the enzyme has greater affinity for ST than for DHST (Table IV). The enzyme failed to utilize the ST analogues HST, EST, and MST (Figure 2) as substrates.

Enzyme activity measurements carried out with the oxidoreductase using OMST or DHOMST as a substrate also demonstrated that both are equally good substrates for the enzyme (Table V). However, the aflatoxin B_1 precursor OMST is the preferred substrate for the enzyme over the aflatoxin B_2 precursor DHOMST. Also, HOMST and MST (Figure 2) were not utilized as substrates by the oxidoreductase.

Substrate Competition Studies with the Enzymes in the Final Steps of Aflatoxin Synthesis. Since the aflatoxin B_2 precursors were found to be substrates for the enzymes involved in aflatoxin B_1 synthesis, substrate competition studies were conducted to determine if the AFB_1 and AFB_2 precursors did indeed compete for the same active site of the two enzymes. Substrate competition studies with MT using ST and DHST as substrates demonstrated that the ST to OMST and the DHST to DHOMST reactions were catalyzed by a common enzyme. At a fixed nonsaturating concentration of ST, an increase in DHST concentration caused an exponential decrease in OMST formation (Figure 4A) with a corresponding exponential increase in DHOMST formation until DHST became saturating (nearly 200 μM). Also, at a fixed nonsaturating concentration of DHST, the DHOMST formation

Table V: Kinetic Parameters for the Oxidoreductase with *O*-Methylsterigmatocystin (OMST) or Dihydro-*O*-methylsterigmatocystin (DHOMST) as Substrates[a]

substrate	app K_m (μM)	V_{max} [μmol/(min·mg)]
OMST	1.2 ± 0.3	38.4 ± 6.6 (AFB_1)[b]
DHOMST	13.4 ± 2.7	25.7 ± 6.1 (AFB_2)[b]

[a] K_m and V_{max} were determined by Lineweaver–Burk plots. Reactions were performed for 10 min as described under Materials and Methods by using a fixed, saturating concentration of NADPH (500 μM). The concentrations of OMST and DHOMST were varied from 0.1 to 25 μM and 2 to 150 μM, respectively. [b] Product detected; AFB_1 and AFB_2, aflatoxins B_1 and B_2, respectively.

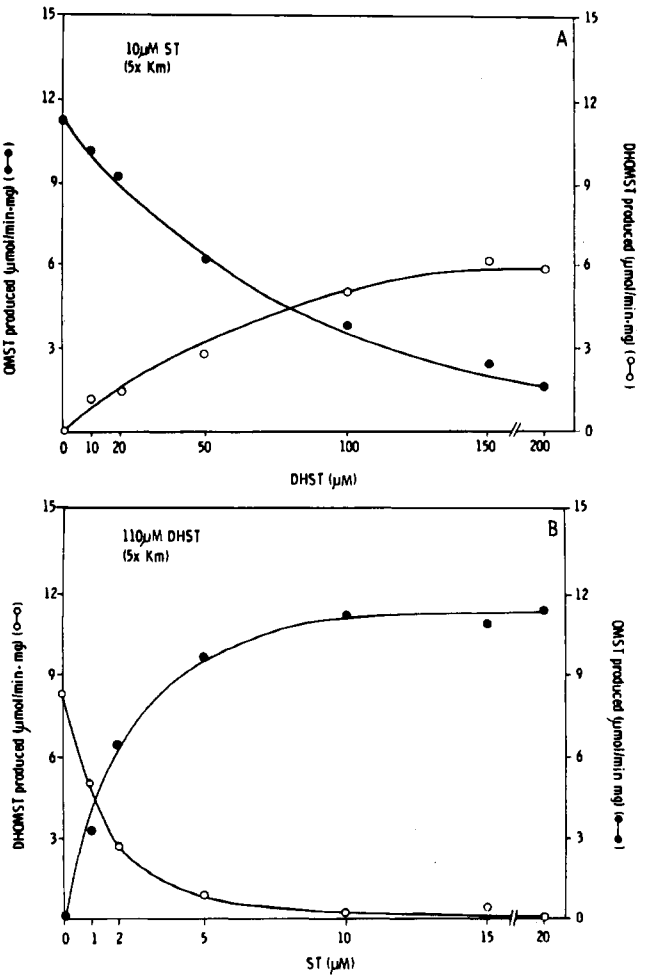

FIGURE 4: Substrate competition studies with the methyltransferase enzyme. (A) Sterigmatocystin (ST) was kept constant in the reaction medium at 10 μM, and dihydrosterigmatocystin (DHST) was varied from 0 to 200 μM; (B) DHST was kept constant at 110 μM, and ST was varied from 0 to 20 μM in the incubation medium. The reactions were carried out for 10 min, and the *O*-methylsterigmatocystin (OMST) (●) and dihydro-*O*-methylsterigmatocystin (DHOMST) production (o) was recorded as described under Materials and Methods.

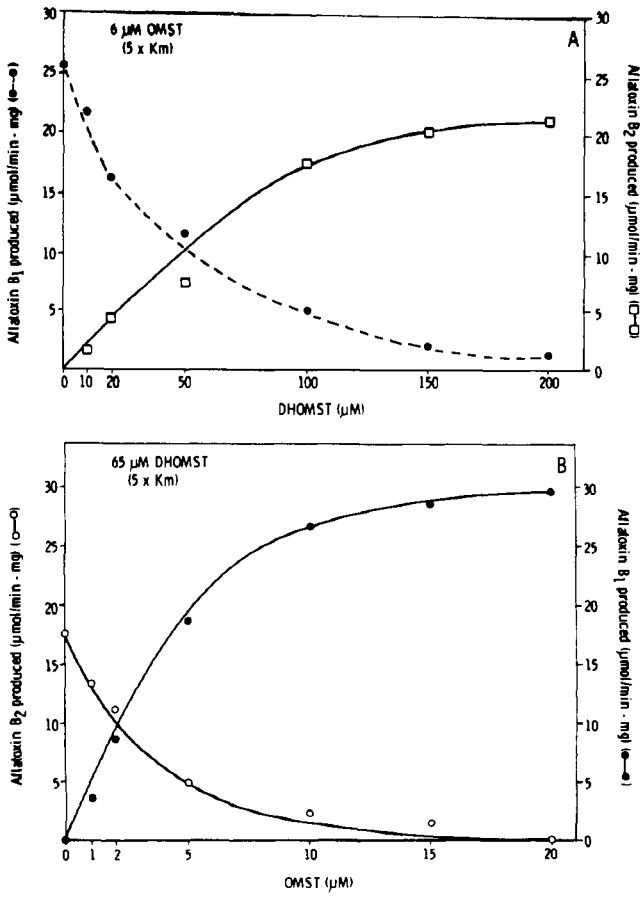

FIGURE 5: Substrate competition studies with the oxidoreductase enzyme. (A) Dihydro-*O*-methylsterigmatocystin (DHOMST) was varied from 0 to 200 μM in the incubation medium at a constant O-methylsterigmatocystin (OMST) (6 μM); (B) DHOMST was kept constant at 65 μM in the reaction medium, and OMST concentration was varied from 0 to 20 μM. The reactions were carried out for 10 min, and the AFB_1 (●) and AFB_2 (○) produced were determined as described under Materials and Methods.

decreased exponentially with an increase in ST concentration (Figure 4B), and a corresponding increase in OMST formation was detected.

Substrate competition studies with the oxidoreductase using OMST and DHOMST as substrates also demonstrated that AFB_1 and AFB_2 formation from OMST and DHOMST is catalyzed by the same enzyme. An increase in DHOMST concentration, at a fixed concentration of OMST (nearly 5 × K_m), caused an exponential decrease in AFB_1 formation (Figure 5A) with a corresponding increase in AFB_2 formation until DHOMST concentration became saturating (nearly 150 μM). Similarly, at a fixed nonsaturating DHOMST concentration, AFB_2 formation decreased exponentially with an increase in OMST concentration, and a corresponding increase in AFB_1 formation was observed (Figure 5B).

The results clearly indicate that the late stages of AFB_1 and AFB_2 synthesis are catalyzed by the same enzymes, which use separate precursors as substrates for the synthesis of each of the toxins.

Similar substrate competition studies with HST and HOMST, which are converted to AFB_2 by intact fungal cells, demonstrated that neither of these metabolites, which are analogues of AFB_1 precursors (ST and OMST, respectively), were substrates of the MT or OR, respectively. HST was not converted to HOMST by the MT, and HOMST was not converted to AFB_2 by the OR. When enzyme assays were conducted with both MT and OR in the incubation medium and the assays were initiated by the addition of either HST or HOMST (200 μM), no aflatoxin formation was observed. Also, HST and HOMST failed to inhibit the utilization of ST/DHST by the MT or OMST/DHOMST by the OR, even at concentrations from 25 to 300 μM in the reaction medium. Therefore, HST and HOMST are not substrates for the MT and OR, respectively. In addition, HST cannot be methylated to HOMST, and HOMST cannot be converted to AFB_{2a} (Figure 2).

Discussion

Biotransformation experiments with blocked mutants of *A. parasiticus* deficient in toxin formation and ^{14}C-radiotracer studies have suggested that AFB_1 and AFB_2 are synthesized by separate chemical pathways (Elsworthy et al., 1970; Dutton et al., 1985; Cleveland et al., 1987a; Cleveland, 1989; Maggon et al., 1979). In the branched AFB_1 and AFB_2 biosynthetic pathway shown in Figure 1, the biosyntheses diverge beginning at the metabolic precursor versiconal hemiacetal acetate. The chemical intermediates placed in the AFB_1 pathway have been demonstrated to be in the following sequence: versicolorin A (Lee et al., 1976) → sterigmatocystin (Hsieh et al., 1973) → *O*-methylsterigmatocystin (Bhatnagar et al., 1987) → AFB_1. Versicolorin C has been postulated to be a precursor of AFB_2 (Dutton et al., 1985) after the metabolic precursor VHA, which is common to both AFB_1 and AFB_2 syntheses. The late stages of AFB_2 synthesis include dihydrosterigmatocystin → dihydro-*O*-methylsterigmatocystin → AFB_2 (Yabe et al., 1988; Cleveland et al., 1987a; Cleveland, 1989). The newly discovered AFB_2 precursors, DHST and DHOMST, have been found in extracts of *Aspergillus* mycelia (Steyn et al., 1980; Cole & Kirksey, 1979; Hatsuda et al., 1972; Cleveland, 1989).

The compounds HST and HOMST (Figure 2), hydrated analogues of ST and OMST, respectively, are also converted to AFB_2 (Table II). AFB_{2a}, a hydrated analogue of AFB_1, was not converted to AFB_1 by whole cells of *A. parasiticus* (Table II). The hydrated compounds are synthesized by derivatization of ST, OMST, and AFB_1, respectively, with TFA (Association of Official Analytical Chemists, 1984), with hydration of the vinyl ether double bond between C_1 and C_2 of the parent compounds under acidic conditions (Figure 2). Acidification of the inner aqueous phase in mycelial cells is taking place during fermentation since the pH of the incubation medium changes from 5.5 to 2.5 during 2–3 days of fungal growth. The hydrated analogues of ST, OMST, and AFB_1 have been extracted from mycelial cultures of *A. parasiticus* (Dutton & Heathcote, 1968; Cleveland et al., 1987a). The metabolites HST, HOMST, and AFB_{2a} appear to act as intermediates between the dihydro- (i.e., AFB_1 precursors) and the tetrahydro- (i.e., AFB_2 precursor) bisfuran series (Figure 6). Anderson and Chung (1990) have identified an NADPH-requiring enzyme activity (reductase) that converts HST and HOMST to DHST and DHOMST, respectively, the same enzyme that converts Ver AH to Ver C (Figure 6). Earlier, Dutton (1988) had postulated that the conversion of HST and HOMST to DHST and DHOMST may be catalyzed by a dehydrogenase. We have demonstrated that AFB_{2a} is not converted to AFB_2 (Table II). However, Dutton (1988) has reported that AFB_{2a} was converted to AFB_2 by cultures of *A. flavus* (SRRC 141), which normally accumulate large amounts of AFB_2 (Table II); no data on detection of this conversion were provided in that report. On the basis of the current observations, the enzyme that converts HST to DHST or HOMST to DHOMST is sensitive to the xanthone moiety of the molecule, since HMST was not utilized by this enzyme. AFB_{2a} may, therefore, also not be a substrate for the enzyme; AFB_{2a} is probably an artifact of acidic conditions, as had been

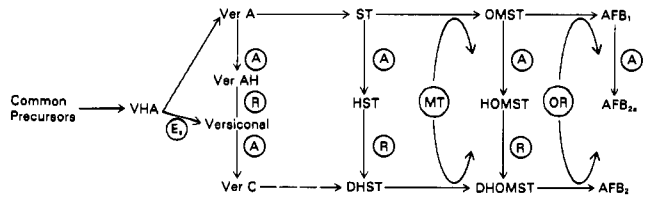

FIGURE 6: Metabolic grid describing the late stages of aflatoxin B_1 and B_2 biosynthesis. Solid lines indicate the reactions experimentally verified in this paper or earlier studies, and the dotted lines indicate postulated reactions. The abbreviations are VHA, versiconal hemiacetal acetate; Ver A, versicolorin A; Ver C, versicolorin C; Ver AH, versicolorin A hemiacetal; versiconal, versiconal hemiacetal alcohol; ST, sterigmatocystin; HST, sterigmatocystin hemiacetal; DHST, dihydrosterigmatocystin; OMST, *O*-methylsterigmatocystin; HOMST, *O*-methylsterigmatocystin hemiacetal; DHOMST, dihydro-*O*-methylsterigmatocystin; AFB_1, aflatoxin B_1; AFB_2, aflatoxin B_2; and AFB_{2a}, aflatoxin B_1 hemiacetal. The reactions are catalyzed by A, acid; E_s, esterase; R, reductase; MT, methyltransferase; and OR, oxidoreductase.

postulated earlier by Dutton and Heathcote (1968).

With the recent identification of the enzymes, a methyltransferase and an oxidoreductase, involved in the late stages of the AFB_1 biosynthetic pathway (Cleveland et al., 1987b) and the subsequent purification of the methyltransferase to homogeneity (Bhatnagar et al., 1988), studies reported here were conducted to ascertain the role of these enzymes in AFB_2 biosynthesis. An attempt was, therefore, made to purify the oxidoreductase for such a study. The enzyme was purified nearly 150-fold by a four-step purification scheme. The enzyme eluted from a gel-filtration column (Figure 3) at a molecular mass between 180 and 200 kDa and is a large protein like the MT (168 kDa). The protein may be composed of smaller molecular mass subunits like the MT (with subunits of 110 and 58 kDa). The enzyme could possibly be a protein complex since it probably catalyzes a very complex reaction (Bhatnagar & Cleveland, 1988; Bhatnagar et al., 1989) involving parahydroxylation, demethylation, reduction, ring cleavage, and conversion of a six-membered ring to a five-membered ring. Dutton (1988) in his review of enzymes in aflatoxin biosynthesis has postulated that the "oxidoreductase" may be composed of at least two enzymes, one of which is likely to be a monooxygenase requiring NADPH and the other a dioxygenase requiring the presence of ferrous ions. The enzyme purified in this study does require NADPH for activity [also, Cleveland and Bhatnagar (1987)]; however, the presence of the ferrous ion was not found to be stimulatory for the activity of the oxidoreductase at this stage of its purification. The oxidoreductase has not been purified to homogeneity, since the denaturing polyacrylamide gel electrophoresis did not exhibit a single protein band. Further purification of this enzyme using affinity chromatography is presently being undertaken in this laboratory and will clarify the composition and the cofactor requirements of this enzyme(s).

Studies with a homogeneous preparation of the MT and the partially purified preparation of the OR clearly demonstrate that these enzymes, which catalyze the late stages of AFB_1 biosynthesis (Cleveland et al., 1987b; Bhatnagar et al., 1988), also catalyze the conversion of DHST to AFB_2 through the DHOMST intermediate (Figure 6). However, ST and OMST are the preferred substrates over DHST and DHOMST for the MT and OR, respectively (Tables IV and V). The results support the observed elevated levels of AFB_1 relative to AFB_2 that routinely occur in cultures of wild-type strains of *A. flavus* and *A. parasiticus* (Leaich & Papa, 1974; Diener & Davis, 1966). Since hemiacetal analogues of ST and OMST do not bind to the active site of these two enzymes, substitutions at the C_1 and C_2 positions of the metabolites (Figure 3) are critical for enzyme specificity. Another analogue of ST, sterigmatocystin ethoxy acetal, was not a substrate for the MT; the results support the contention that substitutions at the C_1 and C_2 positions other than the dihydro substitutions are not accommodated within the active site of the MT and OR enzymes.

The current study clearly demonstrated that although aflatoxins B_1 and B_2 are synthesized by independent processes, their terminal biosynthesis is carried out by the same enzymes. The results also demonstrate that there exists a biosynthetic relationship between the AFB_1 and AFB_2 precursors. Some of the interconversions have been postulated in earlier reports. However, the current observations explicitly elucidate the various biochemical steps and their interactions in the late stages of aflatoxin biosynthesis. Since the first report of the development of a *Aspergillus* mycelial cell-free system (Raj et al., 1969), the MT and OR (our laboratory) and an esterase (Hsieh et al., 1989) have been the only enzymes in the pathway that have been purified thus far. The biochemical similarities between aflatoxin B_1 and B_2 biosyntheses could eventually be exploited in the understanding of the genetic basis for their syntheses as well as in the development of biotechnological approaches for the simultaneous control of the production of these toxins by the fungi. A cDNA library has been constructed from mRNA isolated from *A. parasiticus* mycelia during the onset of AFB_1 biosynthesis (Cleveland & Bhatnagar, 1988) for the purpose of identifying the genes responsible for the methyltransferase and oxidoreductase (Bhatnagar et al., 1989), enzymes specific to aflatoxin biosynthesis.

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A Transforming Growth Factor β (TGF-β) Receptor from Human Placenta Exhibits a Greater Affinity for TGF-β2 than for TGF-β1[†,‡]

E. Jane Mitchell* and Maureen D. O'Connor-McCourt
Receptor Group, National Research Council of Canada, Biotechnology Research Institute, 6100 Royalmount Avenue, Montreal, Quebec H4P 2R2, Canada

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ABSTRACT: Affinity-labeling techniques have been used to identify three types of high-affinity receptors for transforming growth factor β (TGF-β) on the surface of many cells in culture. Here we demonstrate that membrane preparations from tissue sources may also be used as an alternative system for studying the binding properties of TGF-β receptors. Using a chemical cross-linking technique with ^{125}I-TGF-β1 and ^{125}I-TGF-β2 and bis(sulfosuccinimidyl)suberate (BS^3), we have identified and characterized two high-affinity binding components in membrane preparations derived from human term placenta. The larger species, which migrates as a diffuse band of molecular mass 250-350 kDa on sodium dodecyl sulfate-polyacrylamide electrophoresis gels, is characteristic of the TGF-β receptor type III, a proteoglycan containing glycosaminoglycan (GAG) chains of chondroitin and heparan sulfate. The smaller species of molecular mass 140 kDa was identified as the core glycoprotein of this type III receptor by using the techniques of enzymatic deglycosylation and peptide mapping. Competition experiments, using ^{125}I-TGF-β1 or ^{125}I-TGF-β2 and varying amounts of competing unlabeled TGF-β1 or TGF-β2, revealed that both the placental type III proteoglycan and its core glycoprotein belong to a novel class of type III receptors that exhibit a greater affinity for TGF-β2 than for TGF-β1. This preferential binding of TGF-β2 to placental type III receptors suggests differential roles for TGF-β2 and TGF-β1 in placental function.

Transforming growth factor β (TGF-β)[1] represents a family of multifunctional regulatory proteins involved in cellular differentiation and proliferation. TGF-β1 and TGF-β2 have been isolated from tissue sources, whereas the amino acid sequences of TGF-β3, TGF-β4, and TGF-β5 have been deduced from cDNA clones. TGF-βs, as with other polypeptide hormones, appear to act by binding to specific cell surface

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* Author to whom correspondence should be addressed.

[1] Abbreviations: TGF-β, transforming growth factor β; GAG, glycosaminoglycan; BS^3, bis(sulfosuccinimidyl) suberate; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; PBS, Dulbecco's phosphate-buffered saline; BSA, bovine serum albumin; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; EDTA, disodium ethylenediaminetetraacetate; STI, soybean trypsin inhibitor; TCA, trichloroacetic acid; PMSF, phenylmethanesulfonyl fluoride; hCG, human chorionic gonadotropin; hPL, human placental lactogen.

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