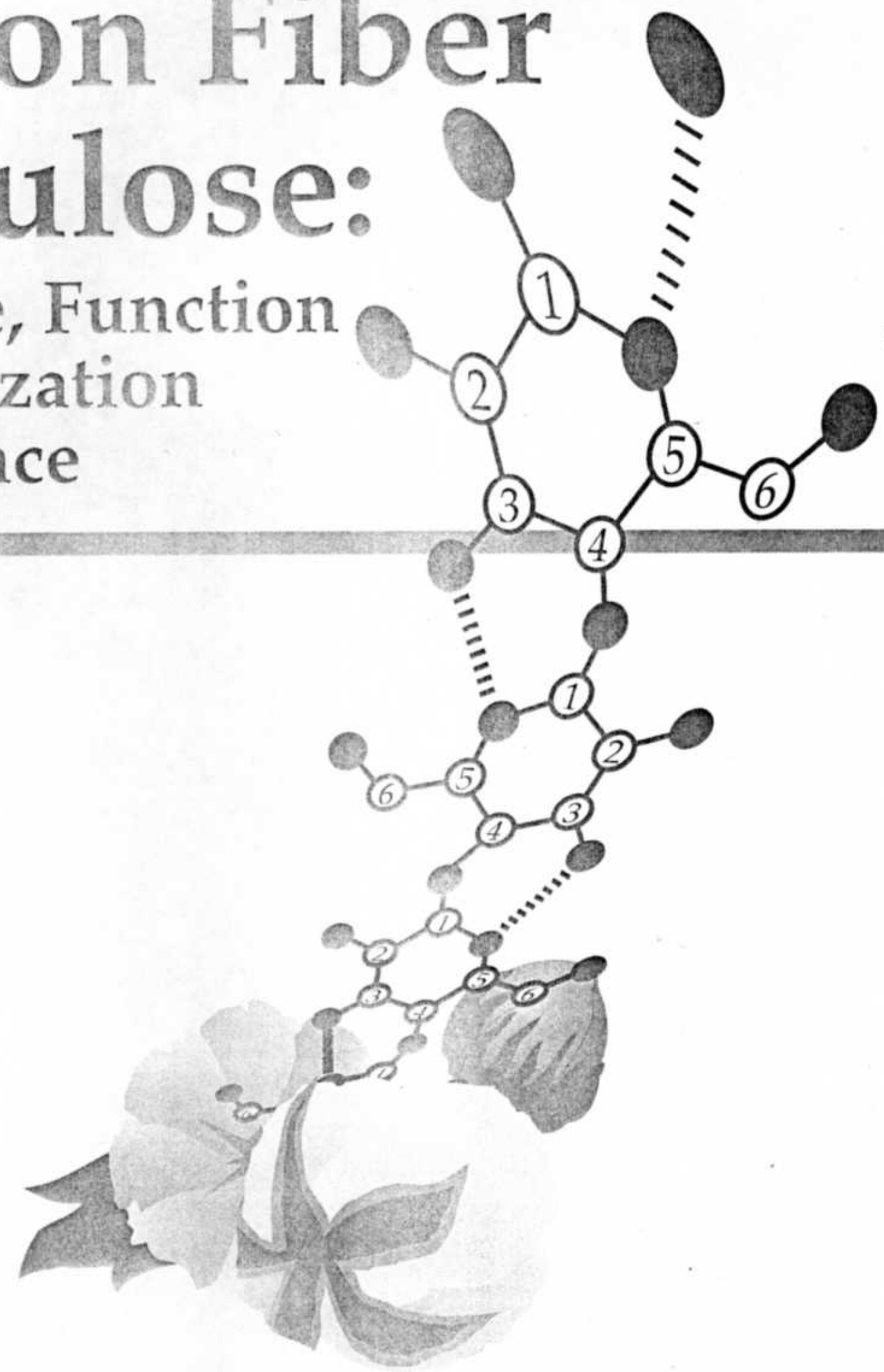
Cotton Fiber Cellulose:

Structure, Function and Utilization Conference



October 28 - 31, 1992 • Savannah, Georgia

Conference Sponsored by

National Cotton Council • Cotton Incorporated • USDA,ARS

Conference registration and proceedings sponsored by a grant
to The Cotton Foundation by Monsanto Corporation

1992 Proceedings

Cotton Fiber Cellulose: Structure, Function & Utilization Conference

October 28-31, 1992

Savannah, Georgia

National Cotton Council of America

National Cotton Council

P. O. Box 12285, Memphis, TN 38182, 901/274-9030

Photoaffinity Labeling of Cellulose Synthases

Richard R. Drake[1], Likun Li[2], and R. Malcolm Brown, Jr.[2]
[1] Department of Biochemistry and Molecular Biology
University of Arkansas for Medical Sciences, Little Rock, AR
[2] Department of Botany, University of Texas, Austin, TX

Abstract

An overview of the synthesis and uses of the photoaffinity analog 5-azido-UDP-glucose in the study of β-glucan synthases from cotton and other systems is presented. When combined with protein purification techniques and enzymatic activity assays, photoaffinity labeling with [β-^{32}P]5-azido-UDP-glucose has allowed the identification of the UDP-glucose catalytic subunits of cellulose synthase from *Acetobacter xylinum* and *Gossypium hirsutum*. An 83 kDa protein was identified as the cellulose synthase from *Acetobacter xylinum* preparations. A 37 kDa protein has been identified as a component of cellulose synthase from cotton, as well as a 52 kDa protein identified as being involved in callose biosynthesis. The criteria by which these proteins were identified and how this type of photoaffinity approach can be utilized in the study of cellulose biosynthesis are discussed.

Introduction

In general, membrane-associated glycosyltransferases are difficult to purify and characterize due to their inherent instability after solubilization from their native membrane environment[5]. Specifically, this observation has been consistently true in the purification and studies of plant β-glucan synthases (1). A photoaffinity analog of UDP-Glc, 5-azido-UDP-Glucose ($5N_3$UDP-Glc) (2,3) has been utilized in the study of yeast, fungal, bacterial and plant β-glucan synthases (3-8). A major advantage to using $5N_3$UDP-Glc in the study of β-glucan synthases has been that the enzymes do not have to be purified to homogeneity to be characterized (3). To demonstrate the effectiveness of a photoaffinity analog, three basic criteria should be met: 1) saturation of active-site photoincorporation; 2) inhibition of photoincorporation by the natural substrate; and 3) show specific photoincorporation in crude enzyme preparations. Presented herein is an overview of studies done with [β-^{32}P]$5N_3$UDP-Glc photolabeling of cellulose synthases from *Acetobacter xylinum* and *Gossypium hirsutum* (6,8). The experiments done to demonstrate the fulfillment of the above photoaffinity criteria for [β-^{32}P]$5N_3$UDP-Glc and cellulose synthases are described.

Materials and Methods

Materials

All enzymes, nucleotides and chromatography resins were purchased from Sigma Chemical Co. All organic reagents were purchased from Aldrich Chemical Co. ^{32}P was purchased from ICN Radiochemicals. Plastic-backed, cellulose thin layer chromatography (TLC) sheets were from Eastman Kodak, and PEI-cellulose plates from EM Science. It is recommended that UDP-glucose pyrophosphorylase purified from yeast (Sigma) and ammonium bicarbonate (Fisher) be used for best results.

Preparation of Cellulose Synthases

The isogenic cotton strain *Gossypium hirsutum* Texas marker 1 were grown and cellulose synthase purified as described by Li and Brown (8). Culturing and harvesting of *Acetobacter xylinum*, strain ATCC 53582, and the resulting purification of cellulose synthase was done as described by Lin *et al.* (6).

Synthesis of $5N_3UMP$

Since the enzymes and the procedure to produce 5-azido-UDP-Glc are commerically available, the chemical syntheses of the precursors $5N_3UMP$ and $5N_3UTP$ are the critical steps in this synthesis and are summarized in Figure 1. $5N_3UMP$ was synthesized as described for $5N_3dUMP$ by Evans and Haley (9) using the following reactions. To the free acid of UMP dissolved in dimethylformamide was added nitrosonium tetrafluoroborate to produce 5-nitro-UMP ($5NO_2UMP$). 5-amino-UMP ($5NH_2UMP$) was produced by reduction of $5NO_2UMP$ with zinc/HCl in water. This reduction step and the subsequent isolation of product has been observed to be the most critical step in this synthetic protocol. 5-azido-UMP was produced by treatment of $5NH_2UMP$ with nitrous acid followed by azide exchange with sodium azide. All reactions were monitored by the shifts in the characteristic UV absorbances of both compounds. At pH 1, $5NO_2UMP$ has a λ_{max} at 302 nm, $5NH_2UMP$ has a λ_{max} of 265 nm and $5N_3UMP$ has a λ_{max} at 288 nm. $5NH_2UMP$ was separated from unreacted $5NO_2UMP$ on a Dowex 50W H^+ column (1 X 26 cm) equilibrated in water. $5N_3UMP$ was purified on a DEAE-cellulose column (2 X 10 cm) equilibrated in 10 mM NH_4HCO_3 and was eluted isocratically with 200 mM NH_4HCO_3. The concentration of $5N_3UMP$ was determined by its absorbance at 288 nm using a molar extinction coefficient of 7600 $M^{-1}cm^{-1}$ (9). Photoreactivity of $5N_3UMP$ was determined by UV-irradiating a small portion in a cuvette, run a UV-spectra and look for loss of absorbance at 288 nm. Generally, yields of 0.2 millimoles $5N_3UMP$ from 0.5 millimoles UMP can be obtained.

Synthesis of $5N_3UTP$

To synthesize $5N_3UTP$ from $5N_3UMP$ and pyrophosphate, the diphenylchlorophosphate coupling procedure of Michelson (10) and a carbonyldiimidazole coupling procedure of Weckbecker and Keppler (11) have been successfully used. For both reactions, the tributylamine salts of $5N_3UMP$ and pyrophosphate were prepared. The carbonyldiimidazole method has produced greater yields and was generally easier to perform. The critical aspects of both of these coupling reactions was to use the most anhydrous chemicals available.

Synthesis of [β-^{32}P]$5N_3UDP$-Glc

[β-^{32}P]$5N_3UDP$-Glc was synthesized by the enzymatic coupling of 5-azido-UTP and [^{32}P]Glc-1-P by yeast UDP-Glc pyrophosphorylase using the following reagents (12). Sucrose phosphorylase, sucrose, $MgCl_2$, sodium phosphate, and 5-10 mCi [^{32}P]P_i are incubated to produce [^{32}P]Glc-1-P. To this [^{32}P]Glc-1-P reaction mixture is added UDP-glucose pyrophosphorylase, inorganic pyrophosphatase, and $5N_3UTP$ (0.5 micromoles) for 1 hour at room temperature. The production of [^{32}P]Glc-1-P and [β-^{32}P]$5N_3UDP$-Glc can be monitored by TLC on cellulose plates developed in methanol:88% formic acid:water (80:15:5) (2,12). Purification of [β-^{32}P]$5N_3UDP$-Glc was done as previously described (12).

Photoaffinity Labeling Procedures

The following is a general outline of the procedures used specifically with [β-^{32}P]$5N_3UDP$-Glc to photolabel membrane-associated glycosyltransferases. The basic photolabeling reaction consists of incubating the photoprobe with an enzyme preparation in a microcentrifuge tube for 10 sec followed by irradiation for 90 sec with a hand-held UV lamp (UVP-11, Ultraviolet Products, Inc.). In general, the buffers and reaction conditions used to assay the enzyme of interest can be utilized for initial photoaffinity experiments. The reactions are terminated by addition of an equal volume of cold 10% trichloroacetic acid and the enzyme(s) precipitated by centrifugation in a standard microcentrifuge. After careful removal of TCA, precipitated enzyme pellets (sometimes not visible) are resuspended in a protein solubilizing mixture containing 4 M urea,

20 mM dithitothreitol, 100 mM Tris, pH 8.0, 4% sodium dodecyl sulfate (SDS), and bromophenol blue. The resuspended protein samples are separated on an SDS-polyacrylamide gel followed by autoradiography. The relative amount of photoincorporation into a particular protein can be determined by densitometric scanning of the autoradiograph. Specific photolabeling conditions for the *A. xylinum* and cotton cellulose synthases have been previously described (6,8).

Results

Photoaffinity Labeling of (1,3)-β-glucan synthases

(1,3)-β-glucan, or callose, is a ubiquitous component of higher plant cell wall polysaccharides and is an important polymer in the wound response process (1). The biosynthesis of this polysaccharide is catalyzed by UDP-Glc: (1,3)-β-glucan synthase, an enzyme long suspected to be a component in plants of a larger, multi-subunit UDP-Glc: (1,4)-β-glucan synthase complex (1). Photolabeling of this enzyme from several sources with [^{32}P]5N$_3$UDP-Glc has been described (7,8,13). When plasma membranes solubilized from carrot were photolabeled with [^{32}P]5N$_3$UDP-Glc, a photoincorporated 57 kDa polypeptide was identified as a potential subunit of the (1,3)-β-glucan synthase (13). Subsequent photolabeling with [^{32}P]5N$_3$UDP-Glc of (1,3)-β-glucan synthase purified from red beet by the procedure of product-entrapment again identified a 57 kDa polypeptide (7). Maximal photolabeling of this 57 kDa polypeptide was correlated with optimal assay conditions, including metal ion effectors, cellobiose and digitonin activation, pH dependence and phospholipase A2 inactivation.

Photoaffinity Labeling of *A. xylinum* Cellulose Synthase

The enzymes responsible for the biosynthesis of cellulose in higher plants have eluded characterization and identification, due to the fact that until recently (8,14,15), *in vitro* synthesis was rarely detectable or assayable (1). For this reason, the gram-negative bacteria *Acetobacter xylinum*, which synthesizes large amounts of pure cellulose, has been used as a model system (1). Using [^{32}P]5N$_3$UDP-Glc, an 83 kDa polypeptide was photolabeled after product-entrapment purification of proteins from *A. xylinum* plasma membranes (5). Photolabeling of this 83 kDa polypeptide was increased by 45% in the presence of cyclic diguanylic acid, a known activator of *A. xylinum* cellulose synthase (16). This system was an excellent model system to study cellulose synthase photolabeling in regards to the stated photoaffinity criteria. Photolabeling of product-entrapped purified cellulose synthase was saturable (apparent K_d= 68 μM) and was inhibited by UDP-Glc (apparent K_d= 82 μM). The similarity in both values illustrates the affinity of [^{32}P]5N$_3$UDP-Glc for the UDP-Glc active-site of this enzyme. Photolabeling in crude extracts was also demonstrated and used to positively identify the 83 kDa protein as the cellulose synthase (16).

Photoaffinity Labeling of Cotton β-Glucan Synthases

Recently in cotton fibers, appreciable levels of *in vitro* synthesis of (1,4)-β-glucan have been reported after using two specific product-entrappment procedures which differentially enrich enzymes responsible for either (1,3)-β-glucan or (1,4)-β-glucan (8). Membrane extracts prepared from cotton have been one of the few higher plant *in vitro* systems in which any (1,4)-β-glucan synthesis has been detected, though until now such activity could not be clearly differentiated from distinct Golgi-localized (1,4)-β-glucan synthases thought to be involved in xyloglucan biosynthesis (1). Photolabeling of the cotton glucan synthase preparations enriched for (1,4)-β-glucan activity resulted in a 37 kDa polypeptide specifically photoincorporating [^{32}P]5N$_3$UDP-Glc. This photolabeling was saturable and inhibited by UDP-Glc with similar apparent Kd values (8). The photolabeling of this 37 kDa protein in these

preparations was directly related to the conditions described for optimal activity (8, 14) including metal ion requirements, pH and guanosine nucleotide effectors.

Using different entrapment conditions which favored (1,3)-β-glucan synthase activity, [^{32}P]5N$_3$UDP-Glc photolabeling identified a 52 kDa polypeptide. Under optimal conditions, [^{32}P]5N$_3$UDP-Glc photolabeling of this 52 kDa protein was saturable and inhibited by UDP-Glc (8). In contrast to the photolabeling of the 37 kDa protein in (1,4)-β-glucan synthase enriched fractions, photolabeling of this 52 kDa protein was dependent on different metal ion requirements and pH optima (8).

Discussion

As these studies have indicated, [^{32}P]5N$_3$UDP-Glc is a very effective tool in identifying and characterizing callose and cellulose synthases. There are several properties of this analog which gives it advantages over other affinity analogs and contribute to its overall usefulness. The major chemical advantage is that upon UV-irradiation, a highly reactive nitrene intermediate is generated with a half-life of milliseconds or less (17). Other photoaffinity analogs which utilize an aryl substituted azide (i.e., 4-azido-2-nitrophenyl or (18)) do not generate nitrene intermediates after photolysis, but longer-lived, more stable dehydroazepene intermediates (3). Another specific advantage of [^{32}P]5N$_3$UDP-Glc is that it contains a radiolabel of high specific activity which is an absolute requirement for rapidly identifying enzymes in crude preparations. Lastly, the only difference between 5-azido-UDP-Glc and UDP-Glc is the 5-azido moiety. This substitution has proven to be minimal in regards to altering recognition of this analog by UDP-Glc utilizing enzymes (3). Moreover, the high similarity to UDP-Glc allows discrimination in photolabeling of UDP-glucosyltransferases to other UDP-glycosyltransferaseses (i.e. those utilizing UDP-Gal or UDP-GlcNAc). This last property is critical to the specificity of photolabeling with [^{32}P]5N$_3$UDP-Glc that has been observed in studies with cellulose synthase and other systems. For these reasons, the base-substituted azido nucleotide analogs have proven to be the most specific and useful photoaffinity analogs for use in biological systems (3,19).

The current studies utilizing [^{32}P]5N$_3$UDP-Glc to examine cellulose and callose synthases illustrate how this analog can be used to identify and characterize membrane-associated UDP-glucosyltransferase catalytic subunits. The identification of the *A. xylinum* cellulose synthase by photoaffinity labeling allowed the subsequent identification of the gene encoding this enzyme and other genes in the cellulose synthase operon (20, 21). A similar goal is currently being pursued with the identification of the photolabeled 37 kDa cotton cellulose synthase. This 37 kDa enzyme was determined to be cellulose synthase by correlating the photoaffinity labeling results to those determined for the enzymatic assays and protein purification results (8, 14, 15). By utilizing enzyme preparations specifically enriched in cellulose synthase activity, a close correlation of the enzymatic K_m for UDP-Glc and the K_d's of saturation and UDP-Glc inhibition of photoincorporation was observed. Other photolabeling conditions including metal ion requirements and pH optima were also varied and correlated to the enzymatic data (8, 14). Similar conditions were varied with the photolabeling of the 52 kDa protein in purified preparations enriched in callose synthase activity.

Taken together, the data indicate that the UDP-Glc catalytic subunits of cellulose and callose synthases in cotton are 37 kDa and 52 kDa proteins respectively. Using direct photo-crosslinking of [α-^{32}P]UDP-Glc of partially purified cotton callose synthase, a 52 kDa protein was also identified as the likely UDP-Glc catalytic subunit (22). In this same study in cotton, a 34 kDa protein was identified by UDP-[^{3}H]pyridoxal affinity labeling (22). This type of chemical affinity labeling is

difficult to interpret since UDP-pyridoxal will not distinguish between UDP-glucosyltransferases and other UDP-glycosyltransferases. It is unlikely that this 34 kDa protein is the 37 kDa protein identified by [^{32}P]5N$_3$UDP-Glc photolabeling since the metal ion requirements were completely different and not consistent with cellulose synthase activity (8, 14). Using entrapment purification and immunological techniques, a 55 kDa protein in peas was identified as callose synthase (23). A glucan synthase complex has been immunoprecipitated from Italian ryegrass (*Lolium multiflorum*) which could synthesize both (1,3)-β-glucan and (1,4)-β-glucan polymers. When this glucan synthase preparation was photolabeled with [^{125}I]-[3-(p-azidosalicylamide)]-allyl-UDP-glucose, a 31 kDa protein was found to be the only subunit in the complex which photoincorporated the analog (18). Additional studies on many other plant systems are necessary, but these data suggest that the UDP-Glc catalytic subunit of (1,4)-β-glucan synthases have molecular masses in the 30-40 kDa range, while the masses of the catalytic subunits of the (1,3)-β-glucan synthases are in the 50-60 kDa range.

With the development of additional azido-nucleoside diphosphate sugar analogs and other azido-nucleotides, similar approaches and techniques as those described herein can be implemented to identify and characterize membrane-associated glycosyltransferases involved in β-glucan and mixed polymer glycan biosynthesis in plants. A general approach could be as follows: 1) establish enzyme activity of the glycosyltransferase; 2) optimize this activity in regards to pH, K_m of substrates, effects of inhibitors and/or co-factors, etc.; 3) prepare the appropriate photoaffinity analog by chemical and/or enzymatic syntheses; 4) use the determined enzyme properties in conjunction with the analog(s) to identify the enzyme of interest; 5) partially purify this enzyme and correlate photolabeled proteins with activity; 6) use preparative gel electrophoresis or another preparative technique to isolate the photolabeled polypeptide; and 7) use this isolated protein to obtain peptide sequence for further uses in immunological and/or molecular genetic techniques. This approach has become more feasible with the introduction of a new preparative gel electrophoresis system from Bio-Rad. This system allows simultaneous elution and collection of the separated proteins in fractions. When combined with photoaffinity labeling, this technique has been used successfully to isolate highly enriched membrane-UDP-glycosyltransferases from partially purified pig liver preparations in quantities suitable for sequencing studies (3).

Literature Cited

1. **Delmer, D.** (1987) Cellulose Biosynthesis. Ann. Rev. Plant Physiol., **38**, 259-290

2. **Drake, R.R., Jr., Evans, R.K., Wolf, M.J., and Haley, B.E.** (1989) 5-Azido-UDP-Glucose: Development of Photoaffinity Probes for Nucleotide Diphosphate Sugar Binding Sites. J. Biol. Chem., **264**, 11928-11933.

3. **Drake, R.R. and Elbein, A.D.** (1992) Photoaffinity Labeling of Glycosyltransferases. Glycobiology, **2**, 279-284.

4. **Frost, D.J., Drake, R.R., and Wasserman, B.P.** (1992) (1,3)-β-Glucan Synthase from *S. Cerevisiae*: In vitro Activation by β-Lactogobulin or Brij-35, and Photoaffintiy Labeling of Enriched Microsomal Fractions with 5-Azido-UDP-Glc and 8-Azido-GTP. *Current Microbiology*, **24**, 295-300.

5. **Beauvais, A. and Latge, J.P.** (1991) Modulation of Glucan and Chitin Synthesis, in Fungal Cell Wall and Immune Response, eds. J.P. Latge and D. Boucias, (Springer-Verlag, Berlin), pp 97-110

6. **Lin, F.C., Brown, R.M., Jr., Drake, R.R., and Haley, B.E.** (1990) Photoaffinity Labeling of Cellulose Synthase in *Acetobacter xylinum* with 5-azido-UPD-glc. J. Biol. Chem., **265**, 4782-4784.

7. **Frost, D.J., Read, S.M., Drake, R.R., Haley, B.E., and Wasserman, B.P.** (1990) Identification of the UDPG-binding Polypeptide of (1,3)-β-Glucan Synthases from Higher Plants by Photoaffinity Labeling with 5-azido-UDP-Glucose. J. Biol. Chem., **265**, 2162-2167.

8. **Li, L., Drake, R.R., Clement, S., and Brown, R.M., Jr.** (1992) β-Glucan Synthesis in the Cotton Fiber, II. Identification of β-Glucan Synthases by Photoaffinity Labeling with [β-^{32}P]5N$_3$UDP glucose. Manuscript submitted.

9. **Evans, R.K. and Haley, B.E.** (1987) Synthesis and Biological Properties of 5-Azido-2'-deoxyuridine 5'-triphosphate, a Photoactive Nucelotide Suitable for Making Light-Sensitive DNA. *Biochemistry,* **26**, 269-276.

10. **Michelson, A.M.** (1964) Synthesis of Nucleotide Anhydrides by Anion Exchange. Biochim. Biophys. Acta, **91**, 1-13.

11. **Weckbecker, G. and Keppler, D.O.R.** (1984) Substrate Properties of 5-Fluorouridine Diphospho Sugars Detected in Hepatoma Cells. Biochem. Pharmacol., **33**, 2291-2298.

12. **Drake, R.R., Zimniak, P., Haley, B.E., Lester, R., Elbein, A.D., and Radominska, A** (1991) Synthesis and Characterization of 5-Azido-UDP-Glucuronic Acid: A New Photoaffinity Probe for UDP-Glucuonic Acid-Utilizing Proteins. J. Biol. Chem., **266**, 23257-23260.

13. **Lawson, G.S., Mason, T.L., Sabin, R.D., Sloan, M.E., Drake, R.R., Haley, B.E., and Wasserman, B.P.** (1989) UDP-glucose: (1,3)-β-glucan Synthase from Daucus Carota: Characterization, Photoaffinity Labeling and Solubilization. Plant Physiology, **90**, 101-108.

14. **Li, L. and Brown, R.M., Jr.** (1992) β-Glucan Synthesis in the Cotton Fiber, I. Regulation and Kinetic Properties of B-Glucan Synthases. Manuscript submitted.

15. **Okuda, K., Li, L., Kudlicka, K., Kuga, S. and Brown, R.M., Jr.** (1992) β-Glucan Synthesis in the Cotton Fiber, III. Product Analysis. Manuscript submitted

16. **Ross, P., Weinhouse, H., Aloni, Y., Michaeli, P., Weinberger-Ohana, P.,Mayer, R., Braun, S., de Vroom, E., van der Marel, G.A., van Boom, J.H., and Benziman, M.** (1987) Regulation of Cellulose Synhtesis in Acetobacter xylinum by cyclic diguanylic acid. Nature, **325**, 279-281.

17. **Bayley, H.** (1983) "Photogenerated Reagents in Biochemistry and Molecular Biology", (Elsevier, New York), pp 2-6.

18. **Meikle, P.J., Ng, K.F., Johnson, E., Hoogenraad, N.J. and Stone, B.A.** (1991) The β-Glucan Synthase from Lolium multiflorum: Detergent Solubilization, Purification using Monoclonal Antibodies, and Photoaffinity Labeling with a Novel Photoreactive Pyrimidine Analogue of Uridine 5'-Diphosphoglucose. *J. Biol. Chem.*, **266**, 22569-22581.

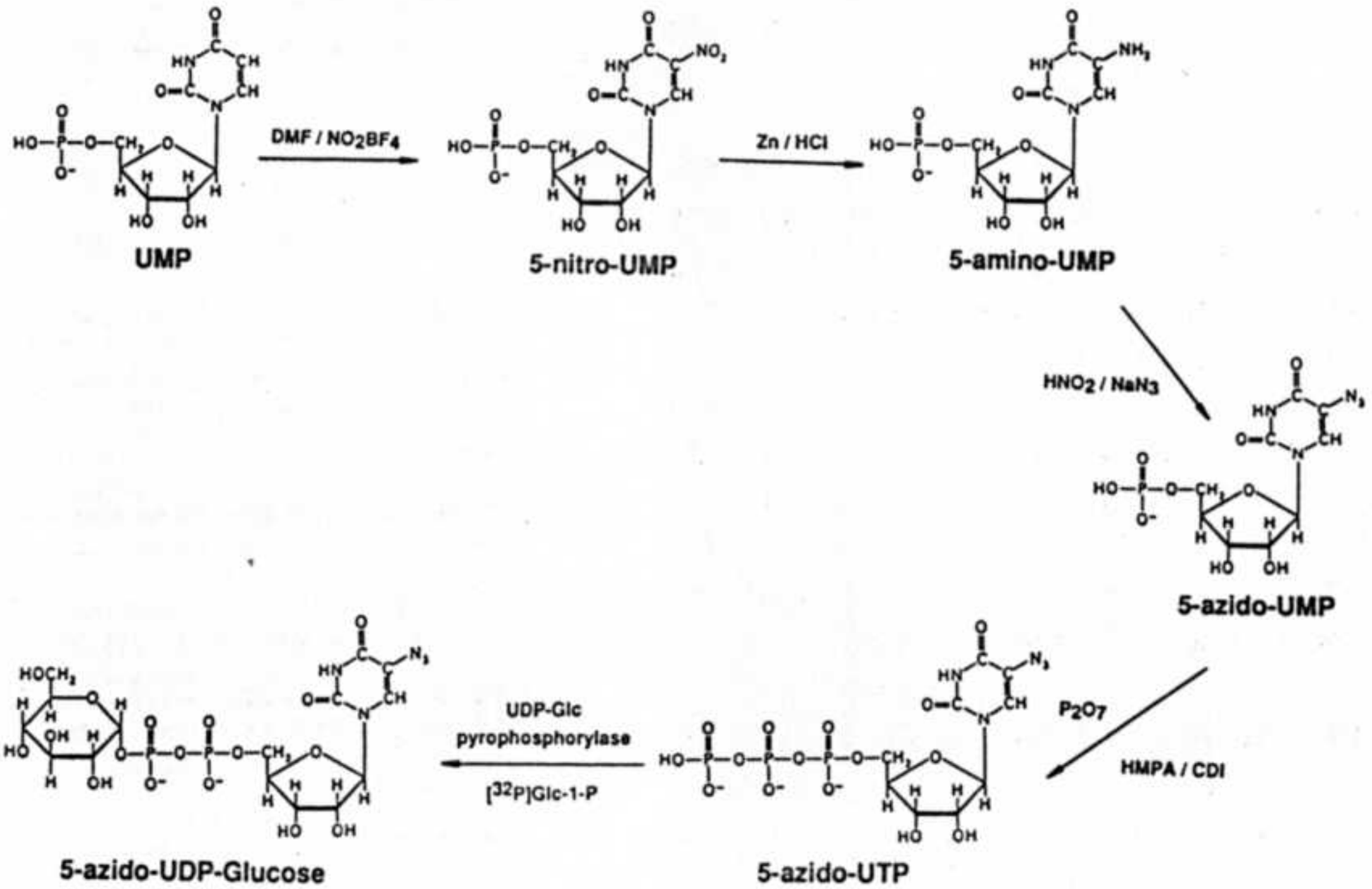
19. **Haley, B.E.** (1991) Nucleotide Photoaffinity Labeling of Protein Kinase Subunits. *Methods in Enzymology*, **200**, 477-487.

20. **Saxena, I.M., Lin, F-C, and Brown, R.M., Jr.** (1990) Cloning and Sequencing of the Cellulose Synthase Catalytic Subunit Gene of *Acetobacter xylinum*. Plant Mol. Biol., **15**, 673-683.

21. **Saxena, I.M., Lin, F-C, and Brown, R.M., Jr.** (1990) Identification of a New Gene in an Operon for Cellulose Biosynthesis in *Acetobacter xylinum*. Plant Mol. Biol., **16**, 947-954.

22. **Delmer, D.P., Solomon, M., and Read, S.M.** (1991) Direct Photolabeling of [^{32}P]UDP-glucose for Identification of a Subunit of Cotton Fiber Callose Synthase. Plant Physiol. **95**, 556-563.

23. **Dhugga, K.S. and Ray, P.M.** (1991) A 55 kDa Plasma Membrane-Associated Polypeptide is Involvedin β-1,3-glucan Synthase Activity in Pea Tissue. FEBS Lett., **278**, 283-286.


UMP
DMF / NO2BF4
5-nitro-UMP
Zn / HCl
5-amino-UMP
HNO2 / NaN3
5-azido-UMP
P2O7
HMPA / CDI
5-azido-UTP
UDP-Glc
pyrophosphorylase
[32P]Glc-1-P
5-azido-UDP-Glucose