Identification of the Uridine 5'-Diphosphoglucohe (UDP-Glc) Binding Subunit of Cellulose Synthase in Acetobacter xylinum Using the Photoaffinity Probe 5-Azido-UDP-Glc

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Photoaffinity labeling of purified cellulose synthase with [β-32P]5-azidouridine 5'-diphosphoglucohe (UDP-Glc) has been used to identify the UDP-Glc binding subunit of the cellulose synthase from Acetobacter xylinum strain ATCC 55382. The results showed an exclusive labeling of an 83-kDa polypeptide. Photoinactivation of [β-32P]5-azido-UDP-Glc is stimulated by the cellulose synthase activator, bis-(3→5') cyclic diguanylic acid. Addition of increasing amounts of UDP-Glc prevents photolabeling of the 83-kDa polypeptide. The reversible and photocatalyzed binding of this photoprobe also showed saturation kinetics. These studies demonstrate that the 83-kDa polypeptide is the catalytic subunit of the cellulose synthase in A. xylinum strain ATCC 55382.

Cellulose is the most abundant macromolecule on earth. It has served the needs of mankind in a variety of ways through thousands of years. Because of abundant production and the purity of its cellulose, a Gram-negative bacterium, Acetobacter xylinum, has become a model system to study the biosynthesis of cellulose (1). UDP-Glc1 has been characterized as a glucosyl donor for cellulose synthesis (2), and c-di-GMP was shown as an activator for the cellulose synthase (3). Recently, two major polypeptides of molecular masses 83 and 93 kDa, present in the partially purified cellulose synthase, have been implicated as components of the cellulose synthase complex in A. xylinum ATCC 55382 (4). Therefore, to identify the catalytic subunit of the cellulose synthase becomes an immediate task for further biochemical characterization of cellulose synthase.

The use of azidonucleotide analogs has proven to be very effective in answering a number of biochemical and biological questions concerning nucleotide binding sites and nucleotide binding proteins which have been difficult to investigate using more conventional techniques (5, 6). Recently, the UDP-Glc analog 5-N,UDP-Glc was synthesized and successfully used to probe a wide variety of UDP-Glc-utilizing enzymes (7) as well as its 1,3-β-glucan synthase of higher plants (8). In this report, [β-32P]5-N,UDP-Glc was used to specifically and effectively photolabel an 83-kDa polypeptide in a purified cellulose synthase preparation from A. xylinum strain ATCC 55382.

EXPERIMENTAL PROCEDURES

Purification of Cellulose Synthase—A. xylinum strain ATCC 55382 was used. The cells were cultured and harvested as previously described (4). The cellulose synthase was purified from detergent-solubilized enzymes using the product entrapment method following a published procedure (4). Three detergents, digitonin, Triton X-100, and n-octyl glucoside, were used to solubilize the enzymes from membrane fractions, respectively. The activator for cellulose synthase, i.e., c-di-GMP, was derived from N-Gx supernatant prepared as described earlier (4).

Photoaffinity Labeling—[β-32P]5-N,UDP-Glc was synthesized by a procedure described by Drake et al. (7). In addition to 20 μl of activator for cellulose synthase, each reaction mixture contained 50 mM Tris-HCl, 10 mM MgCl2, 1 mM CaCl2, 1 mM EDTA, the indicated protein quantity of enzyme sample, and 20 μl of [β-32P]5-N,UDP-Glc (specific activity, 24 Ci/mmol) in a final reaction volume of 50 μl at pH 8.0–8.5. For competition experiments, the competitor, namely UDP-Glc, was added at final concentrations of 0, 50, 100, and 300 μM, respectively. For saturation studies, [β-32P]5-N,UDP-Glc was added at final concentrations of 20, 50, 100, 300, and 500 μM, respectively. Reactions were incubated at 30 °C for 30 min, followed by short wavelength UV irradiation (254 nm) with a hand-held UV lamp (Ultraviolet Products, Inc., model UVSL-58) in open Eppendorf microcentrifuge tubes at a distance of 4 cm for 60 s. Reactions were terminated by the addition of 0.3 ml of 7% perchloric acid. After 20 min, the samples were centrifuged for 3 min in an Eppendorf model 5412 centrifuge. The pellet was suspended in lithium dodecyl sulfate sample buffer for subsequent lithium dodecyl sulfate-polyacrylamide gel electrophoresis as previously described (4). The Coomassie Blue-stained gel was dried and autoradiographed. Autoradiography was performed at ~80 °C with Kodak X-Omat AR film and a Du Pont Cronex intensifying screen. The molecular mass standards (Bio-Rad) were phosphorylase b (97 kDa), bovine serum albumin (66 kDa), ovalbumin (45 kDa), and carbonic anhydrase (31 kDa).

The quantitation of the autoradiographs was measured by gray value discrimination of a digitized image (recorded on a Kontron/Zeiss IBAS image processing system). From these data, the corresponding Kd was calculated.

RESULTS

Two crude enzyme preparations, membranous and digitonin-solubilized forms, and the purified enzyme were tested for their ability to photoincorporate [β-32P]5-N,UDP-Glc (Fig. 1). With or without the presence of c-di-GMP, there was no labeling of the 83-kDa band found in the membrane fraction except for a 57-kDa protein band (Fig. 1, lanes 1 and 2). A similar labeling of the 57-kDa band was observed in the digitonin-solubilized enzyme preparation (Fig. 1, lanes 3 and 4); however, a faintly labeled band corresponding to the 83-kDa polypeptide was seen in the presence of c-di-GMP (Fig. 1, lanes 2 and 4). In the purified preparation, the 83-kDa band became more intensely labeled (Fig. 1, lane 5), and the photoincorporation increased 45% more in the presence of c-di-GMP (Fig. 1, lane 6).

Fig. 2 shows a Coomassie Blue-stained gel with the corresponding autoradiograph, demonstrating photolabeling of the
Fig. 1. Photoaffinity labeling of cellulose synthase in various enzyme samples using 20 μM [β-32P]-5-N UDP-Glc. Samples in lanes 1 and 2 are the membrane fractions, 40 μg of protein/lane. Samples in lanes 3 and 4 are the digitonin-solubilized enzyme (20 μg of protein/lane). Samples in lanes 5 and 6 are the purified cellulose synthase from digitonin-solubilized preparations (3 μg of protein/lane). Either plus (+) or minus (−) 20 μl of cellulose synthase activator contained in the reaction mixture (see "Experimental Procedures") is indicated.

Fig. 2. Coomassie Blue-stained 8% gel (A) and its corresponding autoradiograph (B) of the cellulose synthase in purified fractions prepared by three detergents, digitonin, Triton X-100, and n-octylglucoside, respectively. Samples in lanes 1 and 2 are the enzymes purified from the digitonin-extracted preparations (5 μg of protein/lane). The sample in lane 3 is the enzyme purified from the n-octylglucoside-extracted preparations (2.55 μg of protein). The sample in lane 4 is the enzyme purified from the Triton X-100-extracted preparations (5 μg of protein). The reaction mixture not illuminated with UV light is shown in lane 1.

cellulose synthase in various fractions purified using different detergents. In all cases, the 83-kDa protein band was exclusively labeled with [β-32P]-5-N UDP-Glc (Fig. 2B, lanes 2–4). Control experiments in which the reaction mixtures were not irradiated with UV light (Fig. 2, A and B, lane 1) or in which preirradiated [β-32P]-5-N UDP-Glc was used (Fig. 4, lane 1) yielded no labeling of the 83-kDa polypeptide or any other polypeptides.

UDP-glucose is known as the glucosyl donor for cellulose synthase and was selected as a competitor against photoinsertion of [β-32P]-5-N UDP-glucose. As competitor concentrations increased from 50 to 300 μM in the presence of 20 μM [β-32P]-5-N UDP-Glc, the photoinsertion of the probe into the 83-kDa polypeptide became reduced (Fig. 3)) with a Kₐ of 82 μM. These UDP-Glc protection studies provide further evidence to validate the specificity of the photoaffinity labeling.

A test of a probe's utility for active site studies is the ability to saturate the available binding sites. Fig. 4 shows the saturation of cellulose synthase photo labeling by [β-32P]-5-N UDP-Glc, with a Kₐ value of 68 μM. The photoinsertion of the probe into the 83-kDa protein band showed that saturation was being obtained with [β-32P]-5-N UDP-Glc at concentrations just above 100 μM. This demonstrated that the photoprobe is binding to a limited number of sites and that nonspecific photoinsertion under these conditions was minimal.

The 57-kDa band in the digitonin-extracted enzyme preparations was labeled either by using preirradiated [β-32P]-5-N UDP-Glc solutions (Fig. 5, lane 1) or in the absence of photoactivation by UV irradiation (Fig. 5, lane 2) as well as by normal photolysis (Fig. 5, lane 3). These results indicate that the 57-kDa protein is not interacting with the photoprobe and that labeling is independent of a phototoxic precursor. Furthermore, the labeling of this protein was inhibited in the presence of 500 μM Glc-1-P or Glc-6-P (Fig. 5, lanes 4 and 5). It was not inhibited in the presence of Glc-1,6-P (Fig. 5, lane 6), which is consistent with the properties of phosphoglucomutase (7, 11).

DISCUSSION

There are three major requirements for demonstrating the validity of specific protein binding in photolabeling studies: (a) only a specific protein(s) in an enzyme preparation is photolabeled with the photoprobe; (b) specific photoincorpor-
Photoaffinity Labeling of Cellulose Synthase

The activity at the active site is measured by the ability of the probe to saturate the binding sites and by prevention of photo-labeling with the native substrate at appropriate concentrations; and (c) the photolabeling is dependent on the presence of activating light to exclude, pseudo-photoaffinity labeling.

Our results with the purified cellulose synthase preparation meet all of these criteria. Thus, these studies confirm the earlier suggestion (4) and provide solid evidence that the 83-kDa polypeptide is the catalytic subunit of the cellulose synthase from A. xylina strain ATCC 53582. Further strong evidence is that c-di-GMP substantially increases the photoinsertion of [β-32P]5-N3UDP-Glc with the 83-kDa protein. This cyclic nucleotide has been shown to be a true activator for cellulose synthase in A. xylina (3). The fibrillar structure and cellulosic nature of the in vitro product have been visualized and characterized by electron microscopy (9). Using x-ray diffraction analysis, the in vitro product from purified enzyme preparation2 or membrane fraction (10) was identified as cellulose II.

In the presence of the cellulose synthase activator, a weakly labeled 83-kDa polypeptide in the membrane fraction or the digitonin-solubilized preparation could be detected (Fig. 1, lanes 2 and 4). In contrast, no detectable labeling is observed when no activator is added (Fig. 1, lanes 1 and 3). Again, this demonstrates that c-di-GMP, the specific activator of cellulose synthase, truly increases the affinity of cellulose synthase for UDP-Glc binding and produces a detectable photoactivated labeling of [β-32P]5-N3UDP-Glc. Two possible reasons for the weak labeling of the 83-kDa protein band from these enzyme preparations are as follows. (a) These crude preparations contain trace amounts of the cellulose synthase and therefore would be expected not to label as heavily as the purified enzyme preparation, and (b) the efficiency of photoinsertion as well as protein concentration obviously affects this labeling.

The 57-kDa protein band labeled in the crude membrane fraction and the digitonin-solubilized enzyme preparation are close to the molecular mass previously reported for phosphoglucomutase in the Gram-negative bacteria (11). Though phosphoglucomutase was reported as a soluble enzyme, labeling of a 62–64-kDa protein following incubation with [β-32P]5-N3UDP-Glc has been observed in several membrane preparations (7). This labeling has been attributed to trace levels of [32P]Glc-1-P present in the photoprobe preparations or due to enzymatic hydrolysis of the photoprobe. Whether the 57-kDa protein is a membrane-bound isozyme of phosphogluco-

\[2\] F. C. Lin and R. M. Brown, Jr., unpublished results.

\[3\] R. R. Drake, Jr. and B. E. Haley, manuscript in preparation.

mutase or is an artifact of the membrane isolation technique has not been determined. Our experiments confirm that the 57-kDa protein is a phosphoglucomutase.

In conclusion, by using a photoaffinity labeling method, the substrate binding site of cellulose synthase from A. xylina strain ATCC 53582 was shown to be an 83-kDa polypeptide. It will be of interest to investigate whether the regulatory subunit of cellulose synthase (i.e. c-di-GMP binding subunit) also is the 83-kDa polypeptide or a different subunit. On the other hand, the possible involvement of a 93-kDa polypeptide in cellulose synthase complex cannot be excluded at present since the enzyme complex may consist of nonidentical subunits for various functions in the polymerization and crystallization of cellulose (4). Previously, the 83-kDa polypeptide was characterized as a glycoprotein by lectin affinity chromatography, Schiff-periodate, and fluorescein isothiocyanate-concanavalin A staining analyses (4). It would be worthwhile to observe how this carbohydrate moiety is localized between outer and cytoplasmic membranes since the cellulose synthase activity was found predominantly in the cytoplasmic membrane (10). The demonstration that [β-32P]5-N3UDP-Glc binds to the catalytic subunit of cellulose synthase in A. xylina should provide a powerful tool for future elucidation of the structure, function, and regulation of the cellulose synthase. Furthermore, it will provide a stepping-stone to the gene. It then becomes feasible to sequence this polypeptide and construct a probe to clone the gene.

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REFERENCES