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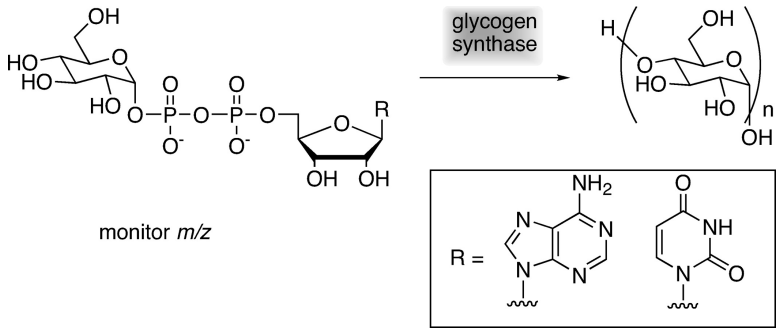

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Discovery of the Archaeal Chemical Link between Glycogen (Starch) Synthase Families Using a New Mass Spectrometry Assay

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The chemical structures of carbohydrate polymers range from homopolymers of glucose such as glycogen or cellulose to the complex heteropolymers such as heparin, which serves as an anticoagulant.[1] Many of these polymers, as well as their chemically and physically modified variants, are key industrial and medical materials. In particular, starch and its modified variants are produced on a 20 million ton scale each year for products that range from paper and textile coatings to food additives and pill components.[1b] Starch and its analogue glycogen are biosynthesized by enzymes that have been classified by sequence similarities into two families that have no significant sequence overlap: the animal/fungal glycogen synthases and the plant/bacterial glycogen (starch) synthases.[2] Recent gene sequence analysis of putative archaea enzymes implicate them as a third family that links the structural and functional features of the other two classes.[2] Herein, we present evidence that an archaea glycogen synthase does indeed catalyze the polymerization in a single binding site of both the substrate used by the animal/fungal synthases and the substrate used by the plant/bacterial synthase. In addition, we describe the first rapid electrospray ionization mass spectrometry-based assay to quantify any carbohydrate-polymerizing activity, the first cloning and recombinant expression as well as verification of the putative function of a glycogen synthase from the hyperthermophilic archaea *Pyrococcus furiosus*, and the characterization of several glycogen synthases with the new assay.

Glycogen synthase activity (Figure 1) is usually monitored by the incorporation of radioactive glucose-nucleotide diphosphate into the glycogen polymer after separation of the starting materials and product.[3] This method is powerful when repeated monitoring of natural substrate turnover is required, but the substrate modifications required become cumbersome if a variety of possible alternative substrates are tested. Mass spectrometry could allow the direct detection of substrates and products without prior purification or the use of special chromogenic or radiolabeled components.[4]

Assays for carbohydrate-polymerizing enzymes using mass spectrometry should be feasible because the nucleotide diphosphate sugar substrates of this class of enzymes are readily detectable by electrospray ionization mass spectrometry (ESI-MS) in negative ion mode. To quantify this signal, a calibration curve was constructed with various concentrations of the activated sugars in the reaction mixture and enzyme assays were run in the concentration range where ionization was linear using the commercially available glycogen synthase from rabbit muscle. The disappearance of sugar nucleotide was monitored over time to construct a Michaelis−Menten curve. Nonlinear regression analysis of reactions run in triplicate resulted in a $K_m = 5.9 \pm 0.5$ mM and $V_{max} = 0.340 \pm 0.008$ mM/min, which compares favorably to the previous results from the traditional radioactivity-based assay ($K_m = 0.5-48$ mM).[3b,c] Kinetic values determined by mass spectrometry for the recombinant yeast glycogen synthase ($K_m = 11.4 \pm 0.7$ mM and $V_{max} = 0.419 \pm 0.009$ mM/min or an activity $= 28.6 \pm 0.6$ μmol/min/mg) also matched those found by the radioactivity-based assay ($K_m = 2.1$ mM and activity $= 30.2$ μmol/min/mg).[3d] To verify that the substrate was converted into glycogen and not simply hydrolyzed, the amount of glycogen formed at the end of the reaction was measured using a commercially available coupled assay,[5] indicating that the amount of glycogen formed was equivalent to the amount of UDP-glucose consumed as determined by ESI-MS. Our glycogen synthase assay based on ESI-MS enables detection of submicromolar quantities of substrate, thus requiring even lower concentrations of protein, and is amenable to high throughput screening methods.

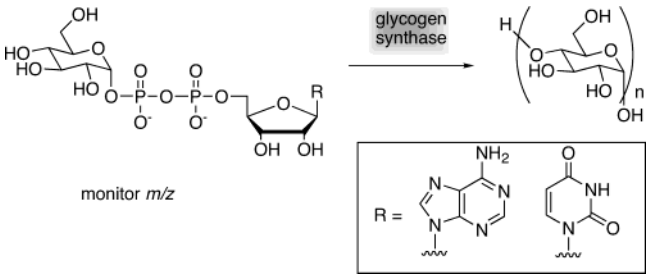

Figure 1. Glycogen/starch synthases catalyze the polymerization of glucose from either adenine- or uridine-diphosphate glucose, depending on the organism. A new assay that allows monitoring of the disappearance of the starting material by electrospray ionization mass spectrometry has enabled the discovery of a glycogen synthase that competitively accepts both adenine- and uridine-nucleotide activated glucose sugars.

Next, the assay was tested with a protein from a putative archaea glycogen synthase gene to verify the gene function and test its chemical links to the characterized synthases from plants, animals, bacteria, and fungi. The genes from hyperthermophiles also might provide thermostable enzymes for synthesis. The recently sequenced thermal vent bacteria *P. furiosus* is of particular interest as a comprehensive structural proteomics effort soon promises a large amount of structural information that will need to be correlated to verified chemical function data.[6] On the basis of primary sequence homology, the gene believed to be responsible for glycogen synthesis in *P. furiosus* was copied using the polymerase chain reaction and ligated into a vector that provides a polyhistidine tag at the C-terminus of the expressed protein for ease of purification. Lac repressor-based protein induction in *Escherichia coli* was started using isobutyl-*C*-galactoside,[7] an improved mimic of isopropyl-thiogalactoside, and the expressed protein was purified using a nickel-affinity column.

To evaluate the function and substrate specificity of this new gene product, the enzyme was first tested with UDP-glucose at varying temperature and pH. A mixture without enzyme was run to control for background substrate hydrolysis at elevated temperatures. The optimal activity was at 80 °C and a pH of 5.0, reaction parameters which are similar to the optimal conditions reported

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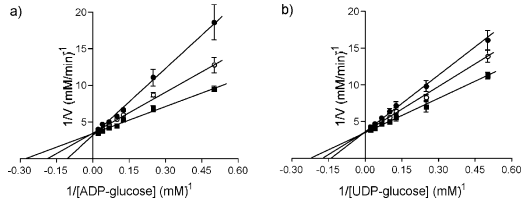

Figure 2. Competition experiments for ADP- versus UDP-glucose for the *P. furiosus* glycogen synthase. Double reciprocal plot of (a) 1/*V* versus 1/[ADP-glucose] at different UDP-glucose concentrations, 0 mM (■), 10 mM (○), 20 mM (●); and (b) 1/*V* vs 1/[UDP-glucose] at different ADP-glucose concentrations, 0 mM (■), 10 mM (○), 20 mM (●).

for the glycogen synthase from *Thermococcus hydrothermalis*[8] and which also would inactivate *E. coli* proteins that might have coeluted with the *P. furiosus* protein. Nonlinear regression analysis of reactions run in triplicate resulted in a $K_m = 4.1 \pm 0.4$ mM and $V_{max} = 0.266 \pm 0.007$ mM/min using UDP-glucose as a substrate. Both the yeast and the rabbit enzymes have substantially enhanced rates in the presence of glucose-6-phosphate, which acts as a regulator.[2,3c,9] However, the *P. furiosus* glycogen synthase shows identical kinetics parameters with or without glucose-6-phosphate present. Surprisingly, this hyperthermophilic enzyme could also turnover ADP-glucose with comparable speed ($K_m = 4.3 \pm 0.3$ mM and $V_{max} = 0.307 \pm 0.007$ mM/min).

This finding raised the intriguing question as to whether the enzyme had a single binding site for such diverse nucleotide substrates or the enzyme was bifunctional with two separate binding sites. Two hyperthermophilic glycogen synthases from *T. hydrothermalis*[8] and *Sulfolobus acidocaldaius*[10] appear to accept both UDP-glucose and ADP-glucose as substrates; however, these enzymes were only partially purified from their natural sources, and therefore the dual activity possibly could be attributed to protein contaminants. Even if a sole enzyme was responsible for the dual activity, no competition studies exist to ascertain if the synthase has two separate binding sites for the nucleotides or if a single binding site competes for such different nucleotide substrates. The former hypothesis makes most sense in light of crystallography studies of several nucleotide-sugar binding proteins that implicate the nucleotide diphosphate portion of the sugar-donor for a significant portion of the substrate binding contacts to the protein.[11] To address this question, competition experiments were carried out with each nucleotide at various concentrations of the other nucleotide in triplicate (Figure 2). These results clearly indicate that UDP-glucose and ADP-glucose serve as competitive inhibitors of one another with a K_i of 18 ± 6 mM for UDP-glucose and 22 ± 2 mM for ADP-glucose. When both substrates are mixed together in equal parts, ADP-glucose was consumed at a slightly faster rate than that of UDP-glucose.

This competition evidence is the first clear indicator that archaea enzymes have a binding pocket that can accommodate both nucleotide-glucose donors commonly found in cells. These hyperthermophilic enzymes then may have lost their ability to bind one or the other nucleotide-sugar as they evolved into the two classes of enzymes found in mesophilic organisms today that accept either ADP- or UDP-glucose as a polymerization substrate. The structural basis of the reduced substrate specificity and the binding requirements of the sugar portion of the donor remain open questions. The reported new ESI-MS-based assay, however, enables the latter question to be addressed without the synthesis of radioactive substrates or development of separation protocols. In addition, this work enables the screening or discovery of new proteins or protein mutants that can polymerize unusual sugars for the biosynthesis of novel carbohydrate polymers.

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Supporting Information Available: Experimental details for production of the *P. furiosus* glycogen synthase, details of the mass spectrometry assays including calibration curves, and all Michaelis−Menten, Lineweaver−Burke, and competition experiment plots (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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