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Examination of the Mechanism of Sucrose Synthetase by Positional Isotope Exchange*

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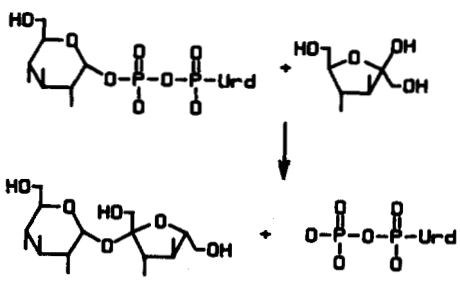
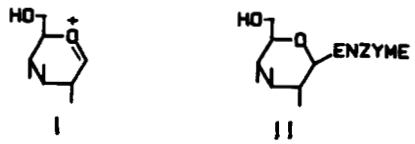
The mechanism of the sucrose synthetase reaction has been probed by the technique of positional isotope exchange. [β-$^{18}O_2$,αβ-^{18}O]UDP-Glc has been synthesized starting from oxygen-18-labeled phosphate and the combined activities of carbamate kinase, hexokinase, phosphoglucomutase, and uridine diphosphoglucose pyrophosphorylase. The oxygen-18 at the αβ-bridge position of the labeled UDP-Glc has been shown to cause a 0.014 ppm upfield chemical shift in the ^{31}P NMR spectrum of both the α- and β-phosphorus atoms in UDP-Glc relative to the unlabeled compound. The chemical shift induced by each of the β-nonbridge oxygen-18 atoms was 0.030 ppm. Incubation of [β-$^{18}O_2$,αβ-^{18}O]UDP-Glc with sucrose synthetase in the presence and absence of 2,5-anhydromannitol did not result in any significant exchange of an oxygen-18 from the β-nonbridge position to the anomeric oxygen of the glucose moiety. It can thus be concluded that either sucrose synthetase does not catalyze the cleavage of the scissile carbon-oxygen bond of UDP-Glc in the absence of fructose or, alternatively, the β-phosphoryl group of the newly formed UDP is rotationally immobilized.

Sucrose synthetase catalyzes the reversible formation of sucrose from UDP-Glc and fructose as indicated below.

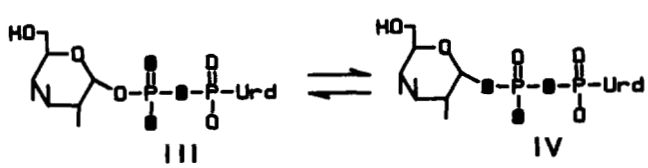


The stereochemical outcome of the overall chemical reaction is thus one of retention of configuration at C-1 of the glucose moiety. This fact would strongly suggest that the carbon-oxygen bond of UDP-Glc is completely broken prior to the nucleophilic attack by the incoming fructose (1). Probable intermediates formed in such a mechanism could either be an oxocarbonium ion (**I**) or a covalent glycosyl-enzyme (**II**) adduct as illustrated below.



However, there is no other independent evidence to suggest that an intermediate is formed in the sucrose synthetase reaction. For example, if a covalent glycosyl-enzyme intermediate was formed, then it might be anticipated that "ping-pong" reaction kinetics would be observed. However, the kinetic mechanism of the enzyme is sequential (2). Therefore, both UDP-Glc and fructose must bind to the protein prior to the release of either product. Thus, if a covalent intermediate is synthesized it is either not formed until fructose binds to the enzyme or, alternatively, the UDP is not released until after fructose binds to the active site. These findings are also supported by the observation that sucrose synthetase from artichoke tubers does not catalyze a sucrose-fructose molecular isotope exchange reaction in the absence of added UDP (3).

The technique of positional isotope exchange (PIX)[1] has been applied to a variety of enzyme-catalyzed reactions in the quest for transient intermediates (4–8). The PIX technique can be used to probe the mechanism of enzymes that direct the attack of nucleophiles at the anomeric center of UDP-Glc. For example, if the carbon-oxygen bond at the anomeric carbon of UDP-Glc is broken prior to the binding of fructose and if the β-phosphoryl group of the resulting UDP is able to rotate, then a positional isotope exchange of a β-nonbridge oxygen and the anomeric oxygen will occur. This exchange reaction is illustrated below (**III** and **IV**) for oxygen-18-labeled UDP-Glc.



The catalysis of such an exchange reaction by sucrose synthetase in the absence of any added fructose would clearly demonstrate that the carbon-oxygen bond of UDP-Glc is broken prior to the binding of the second substrate to the protein and thus conclusively establish the existence of an intermediate.

In this paper we have developed a convenient synthesis for

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[1] The abbreviations used are: PIX, positional isotope exchange; HPLC, high performance liquid chromatography; MES, 2-(*N*-morpholino)ethanesulfonate; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

oxygen-18-labeled UDP-Glc. This molecule can be used to probe for the existence of intermediates in any enzyme-catalyzed reaction that uses UDP-Glc to synthesize more complicated saccharides. The labeled UDP-Glc has been incubated with sucrose synthetase under a variety of experimental conditions in an attempt to demonstrate conclusively the formation of an intermediate.

MATERIALS AND METHODS[2]

RESULTS

The strategy for preparing [β-$^{18}O_2$,$\alpha\beta$-^{18}O]UDP-Glc (**III**) is shown in Scheme I. The synthesis starts with the preparation of [γ-$^{18}O_3$]ATP (**VI**) as catalyzed by carbamate kinase in the presence of ADP and labeled carbamyl phosphate (**V**). The terminal phosphoryl group is then transferred from the ATP to C-6 of glucose in a reaction catalyzed by hexokinase. Phosphoglucomutase is then used to shift the phosphoryl group from C-6 to C-1 of glucose. The final product is formed from UTP and glucose-1-P (**VIII**) in a reaction catalyzed by UDP-Glc pyrophosphorylase in the presence of sufficient pyrophosphatase to pull the reaction to completion. The ^{31}P NMR spectra of the isolated product is shown in Fig. 1*A*. The incorporation of oxygen-18 at the indicated positions is greater than 95%. The upfield chemical shift induced by the incorporation of the oxygen-18 into the α- and β-phosphoryl groups of UDP-Glc is clearly demonstrated in Fig. 1*B*. Fig. 1*B* shows the spectrum of a 1:1 mixture of [β-$^{18}O_2$,$\alpha\beta$-^{18}O] UDP-Glc (**III**) and unlabeled UDP-Glc. The resonance for the α-phosphoryl group is shifted upfield by 0.014 ppm. The resonance for the β-phosphoryl group is shifted upfield by 0.075 ppm. Assuming that the $\alpha\beta$-bridge oxygen induces an identical upfield shift in both the α- and β-phosphoryl groups, then the upfield shift caused by each of the two β-nonbridge oxygens can be calculated as 0.030 ppm.

The occurrence of a positional isotope exchange reaction within UDP-Glc will result in one of the β-nonbridge oxygens interchanging with the anomeric oxygen at C-1 of the glucose moiety (see **III** and **IV**). If ^{31}P NMR spectroscopy is to be used to monitor this reaction, then the upfield chemical shifts induced by the oxygen-18 must be sufficiently different for the two possible orientations. Shown in Fig. 2*A* is the spectrum of [β-$^{18}O_2$,$\alpha\beta$-^{18}O]UDP-Glc (**III**) after incubation with sucrose synthetase and fructose until equilibrium had been reached. The resonance for the β-phosphoryl group of the equilibrated UDP-Glc is now shifted downfield by 0.015 ppm relative to the starting material. This change represents the difference in chemical shift induced by an oxygen-18 in the β-nonbridge position and the C-O-P_β-bridge position. Therefore, the upfield chemical shift on the β-P caused by an oxygen-18 at the C-O-P-bridge position is 0.015 ppm (0.030–0.015). The relative intensities for the resonances of the β-P after equilibration are 2:1. The theoretical value is 2:1, since there are two β-nonbridge oxygens.

If sucrose synthetase is able to cleave the bond between the carbon and the anomeric oxygen of UDP-Glc in the absence of an acceptor cosubstrate and if the β-phosphoryl group of the resulting UDP is able to rotate freely, then the incubation of [β-$^{18}O_2$,$\alpha\beta$-^{18}O]UDP-Glc will result in the appearance of an oxygen-18 atom in the C-O-P-bridging oxygen position (see **III** and **IV**). This occurrence can then be detected by the appearance of a new resonance for the β-P that is 0.015 ppm downfield from the starting material. Shown in Fig. 2*B* is the spectrum of [β-$^{18}O_2$,$\alpha\beta$-^{18}O]UDP-Glc that has been incubated in the presence of 1000 units of sucrose synthetase for 3.25 h at 25 °C and pH 6.0. No positional isotope exchange could be detected. Assuming that the minimum amount of positional isotope exchange that could be detected is 10%, then a maximum value for the exchange rate can be calculated from Equation 1

$$V_{ex} = \frac{-A_0 \ln (1 - F)}{t} \quad (1)$$

where F = fraction of equilibrium value for exchange attained in the UDP-Glc pool at time, t, and A_0 = the concentration of UDP-Glc (4). Similar experiments were conducted at pH 7, 8, and 9, in both the presence and absence of 2,5-anhydromannitol (**XI**). The results are tabulated in Table I.

2,5-Anhydromannitol was tested as an inhibitor of the sucrose synthetase reaction. The inclusion of 1.0 mM 2,5-anhydromannitol in an assay mixture at pH 7.5, containing 1.0 mM UDP-Glc and 4.0 mM fructose, inhibited the rate of UDP formation by 4.3-fold.

DISCUSSION

The timing of the bond cleavage and bond-forming steps in the reaction catalyzed by sucrose synthetase is unknown. The observation of net retention at the anomeric carbon of UDP-Glc and sucrose strongly suggests the formation of some type of intermediate during the transformation from substrates to products. The two most likely intermediates would be an oxocarbonium ion (**I**) and a covalent glycosyl-enzyme adduct (**II**). In either case the carbon-oxygen bond is broken prior to the covalent attack by the incoming fructose. The appropriately oxygen-18-labeled UDP-Glc was synthesized and incubated with sucrose synthetase in an attempt to demonstrate carbon-oxygen bond cleavage prior to the binding of fructose to the active site. The observation of positional isotope exchange from a β-nonbridge oxygen position to the C-O-P-bridge position within UDP-Glc (see **III** and **IV**) in the absence of added fructose would clearly establish the timing and sequence of the bond-breaking step in the mechanism. However, the PIX experiment cannot distinguish between the two alternate intermediates. No positional isotope exchange could be detected (<0.025% of sucrose synthesis rate) at pH values from 6 to 9 when sucrose synthetase was incubated with [β-$^{18}O_2$,$\alpha\beta$-^{18}O]UDP-Glc.

TABLE I

Positional isotope exchange by sucrose synthetase

Enzyme	Time	pH	2,5-Anhydro-mannitol	v_{ex}[a]	v_{chem}[b]/v_{ex}
units	*h*				
1000	3.25	6.0	−	<0.39	>2600
1000	2.25	6.0	+	<0.56	>1800
1000	4.00	7.0	−	<0.31	>3200
1000	5.00	7.0	+	<0.25	>4000
610	5.00	8.0	−	<0.25	>2400
610	4.00	8.0	+	<0.31	>2000
610	2.75	9.0	−	<0.46	>1300
610	1.75	9.0	+	<0.72	>850

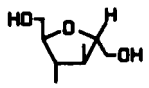
[a] Velocity of positional isotope exchange reaction calculated according to Equation 1 assuming a F value of 0.10.

[b] v_{chem} calculated as the rate of chemical formation of UDP when fructose added at pH 7.5.

[2] Portions of this paper (including "Materials and Methods," Figs. 1 and 2, and Scheme I) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are available from the Journal of Biological Chemistry, 9650 Rockville Pike, Bethesda, MD 20814. Request Document No. 86M-3466, cite the authors, and include a check or money order for $2.40 per set of photocopies. Full size photocopies are also included in the microfilm edition of the Journal that is available from Waverly Press.

There are a number of potential reasons why no PIX was detected with sucrose synthetase. Although bond cleavage and formation are probably discrete chemical steps, the breakage of the carbon-oxygen bond might require fructose at the active site to induce the enzyme into the proper conformational state required for catalysis. In an effort to surmount this potential problem the positional isotope exchange experiments were also conducted in the presence of 2,5-anhydromannitol (**XI**). This molecule is an analog for the β-furanose configuration (**X**) of fructure (13).

X



XI

However, it is lacking the anomeric oxygen and thus cannot be active as a substrate. As indicated under "Results," this compound is a very good inhibitor of the sucrose synthetase reaction and thus it is able to bind to the active site. However, 2,5-anhydromannitol is unable to induce sucrose synthetase to catalyze a PIX reaction at a significant rate. This would indicate that 2,5-anhydromannitol is not a good enough analog for fructose or that the bond cleavage and bond-forming reactions for sucrose synthesis are more tightly coupled than anticipated.

The use of a substrate analog to induce a PIX reaction was first demonstrated by Kokesh and Kakuda (14) with starch phosphorylase. They showed that starch phosphorylase was unable to catalyze the positional isotope exchange of oxygen-18 within glucose-1-P but that significant exchange could be observed when α- or β-cyclodextrin was also included in the reaction mixture. The cyclic structure of the dextrin prevented glycosylation of the dextrin. These results were used to conclude that starch phosphorylase catalyzed the formation of an intermediate prior to the addition of the starch primer.

Restricted rotation of the β-phosphoryl group of UDP would also suppress the detection of a PIX reaction within UDP-Glc. If an intermediate is formed but the β-phosphoryl group is unable to torsionally equilibrate then no PIX will be observed because upon resynthesis of the UDP-Glc the original oxygen-16 will become rebonded to the anomeric carbon. Since no divalent metals are required by sucrose synthetase to coordinate the pyrophosphate linkage in UDP-Glc, the restriction to bond rotation would be required to result from electrostatic interactions to the protein and/or intermediate. At least three examples of restricted bond rotation have been previously proposed by various groups. Farnesyl pyrophosphate synthetase (15), argininosuccinate synthetase (6), and bornyl synthetase (16) have all been shown to catalyze the formation of an intermediate, but in each case a PIX reaction was not observable. If the intermediate formed in the sucrose synthetase reaction is an oxocarbonium ion then it would appear that this cation and UDP are forming a rigid cation-anion pair that is unable to torsionally equilibrate the β-phosphoryl group of UDP.

In summary, UDP-Glc has been enzymatically synthesized with an oxygen-18 label at the nonbridge position of the β-phosphoryl group. Incubation of this compound with sucrose synthetase in the presence and absence of 2,5-anhydromannitol resulted in no significant positional exchange of the anomeric oxygen and the β-nonbridge oxygen. This indicates that either the scissile carbon-oxygen bond is not broken in the reaction mechanism until after the fructose is bound to the enzyme or, alternatively, that the β-phosphoryl group of UDP is unable to rotate at a kinetically significant rate.

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Supplementary Material To

Examination of the Mechanism of Sucrose Synthetase by Positional Isotope Exchange

Amar N. Singh, Leisha S. Hester, and Frank M. Raushel

Materials and Methods

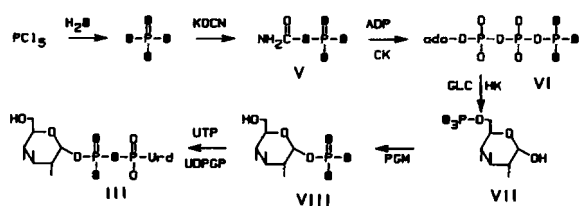
Potassium cyanate was obtained from Alpha Chemical Co. Sodium borohydride and sodium nitrite were purchased from MCB Chemical Co. 2,5-Anhydromannitol was synthesized according to the method of Horton (9) and Bern et al. (10). $KH_2P^{18}O_4$ was synthesized from PCl_5 and oxygen-18 labelled water (97%, Cambridge Isotope Laboratory) according to the method of Risley and Van Etten (11). NMR analysis of the labelled phosphate indicated > 95% incorporation of oxygen-18. All other reagents were acquired from either Sigma or Aldrich.

Preparation of [γ-$^{18}O_3$]ATP (VI). ATP, labelled in the terminal phosphoryl group with oxygen-18, was synthesized by the method of Cohn and Hu (12) with some modification. $KH_2P^{18}O_4$ (300 μmoles) and 2.0 mmoles of KCNO were incubated together in 2.0 mL of 150 mM sodium acetate buffer (pH 4.8). The pH was subsequently maintained at 6.5 with acetic acid. After 30 minutes an additional 0.5 mmoles of KCNO was added and the incubation continued for an additional 15 minutes. At this time ADP (300 μmoles), $MgSO_4$ (300 μmoles), Tris buffer (1500 μmoles, pH 7.5) and 30 units of carbamate kinase were added together in a volume of 2.5 mL. The mixture was incubated at 38 °C until all of the ADP had been converted to ATP. The formation of ATP was monitored by HPLC using a Whatman SAX anion exchange column. The eluting buffer was 0.5 M KH_2PO_4. The synthesis was terminated by centrifugation of the reaction mixture through a CF25 Centriflo ultrafiltration membrane cone (Amicon). The filtrate was diluted to 250 mL and applied to a Whatman DE-52 anion exchange column (1.5 x 50 cm) and eluted with a 2.0 liter gradient of 10-500 mM triethylamine/HCO_3^- buffer, pH 7.5. The fractions containing the ATP were evaporated to dryness. The residue was dissolved in 100 mL of methanol and re-evaporated to dryness to remove any excess triethylamine. The yield was 290 μmoles.

Synthesis of [P-$^{18}O_3$]Glucose-6-P (VII). Hexokinase was used to phosphorylate glucose at C-6 with the oxygen-18 labelled ATP. [γ-$^{18}O_3$]ATP (290 μmoles), glucose (900 μmoles), $MgSO_4$ (300 μmoles), Tris buffer (1500 μmoles, pH 7.5) and 30 units of yeast hexokinase were added together in a volume of 21.5 mL. The formation of glucose-6-P was determined spectrophotometrically with glucose-6-P dehydrogenase. The reaction was terminated after 70 minutes by centrifugation of the reaction mixture through a CF25 Centriflo ultrafiltration membrane cone. The filtrate was diluted to 400 mL and was applied to a Whatman DE-52 anion exchange column (1.5 x 50 cm). The glucose-6-P was eluted using a 2.0 liter gradient of 10 to 200 mM triethylamine/HCO_3^- pH 7.5. The yield was 220 μmoles.

Synthesis of [β-$^{18}O_2$,$\alpha\beta$-^{18}O]Uridine Diphosphoglucose (III). The oxygen-18 labelled UDP-glc was synthesized by the combined activities of phosphoglucomutase and uridine diphosphoglucose pyrophosphorylase. The reaction mixture contained 2.4 mM [P-$^{18}O_3$]glucose-6-P, 5.0 mM UTP, 12.5 mM $MgSO_4$, 200 units of phosphoglucomutase, 100 units of uridine diphosphoglucose pyrophosphorylase, 0.1 mM glucose-1,6-bisphosphate, 100 units of inorganic pyrophosphatase and 250 mM Tris buffer, pH 7.5, in a volume of 40 mL. The formation of UDP-glc was monitored via HPLC. The reaction was quenched by passage of the reaction mixture through an Amicon ultrafiltration membrane cone. The filtrate was diluted to 300 mL and was applied to a Whatman DE-52 anion exchange column. (1.5 x 50 cm). The labelled UDP-glc was eluted with a 2.0 liter gradient of 10 to 250 mM triethylamine\HCO_3^-, pH 7.5. The yield was 73 μmoles.

Scheme I



Positional Isotope Exchange Reactions. Sucrose synthetase was incubated with 0.48 mM [β-$^{18}O_2$,$\alpha\beta$-^{18}O]UDP-glc (III), 50 mM phosphate, and 50 mM MES buffer, pH 6.0, in a volume of 25 mL for various lenghths of time at 25 °C. An identical reaction mixture also contained 0.32 mM 2,5-anhydromannitol (XI). The reaction was terminated by centrifugation of the mixture through a CF25 Centriflo ultrafiltration membrane cone. The filtrate was diluted to 250 mL and the pH was adjusted to approximately 8.0. The UDP-glc was loaded on a Whatman DE-52 anion exchange column (1.5 x 50 cm) and eluted with a gradient of 10 to 250 mM triethylamine/HCO_3^-, pH 7.5. The fractions containing the UDPG were pooled and evaporated to dryness. The residue was dissolved in a 3 mL solution containing 100 mM EDTA, 50 mM phosphate, 150 mM Tris buffer, pH 9.0 and 25% D_2O. Identical experiments were also conducted at pH 7.0, 8.0, and 9.0. The distribution of oxygen-18 was then determined by ^{31}P NMR spectroscopy.

^{31}P Nuclear Magnetic Resonance Measurements. ^{31}P NMR spectra were obtained on a Varian XL-400 multinuclear spectrometer operating at a frequency of 162 MHz. Typical acquisition parameters were 6000 Hz sweeep width, 2.5 second acquisition time, 15 μs pulse width (45°), and broadband proton decoupling. All spectra were referenced to an internal standard of phosphate at pH 9.0. Up to 10,000 transients were collected and Fourier transformed.

Purification of Sucrose Synthetase. Sucrose synthetase was isolated from wheat germ by the method of Mendicino (13) and Marata (14) with some modification. Wheat germ (200 grams) was suspended in 1000 mL of 50 mM phosphate buffer, pH 7.5, and extracted for 4 hours at 4 °C with occassional stirring. The suspension was then centrifuged for 30 min at 6000 rpm. The supernatant solution was brought to 30% saturation with solid ammonium sulfate (0.164 g/mL). The suspension was stirred for 30 minutes and then centrifuged. The supernatant solution was brought to 40% saturation by the slow addition of solid ammonium sulfate (0.056 g/mL) and centrifuged after stirring. The precipitate was dissolved in 200 mL of 25 mM phosphate buffer, pH 7.0, and then 0.258 g/mL of solid ammonium sulfate was added to reprecipitate the protein. This process was repeated three times. The final precipitate was dissolved in 200 mL of 25 mM phosphate buffer pH 7.0, and dialyzed against 2000 mL of the same buffer for 12 hours. The dialyzed solution of protein was centrifuged for 30 minutes at 6000 rpm to remove any insoluble materials. One half of the protein solution was loaded on a Whatman DE-52 anion exchange column (3.0 x 55 cm) and eluted with a linear gradient of 25 to 500 mM phosphate buffer, pH 7.0. The fractions were analyzed for sucrose synthetase activity, pooled, and concentrated using an Amicon PM-30 ultrafiltration membrane. The concentrated solution of protein was loaded on an Ultragel AcA-34 molecular seive column (5.0 x 154 cm) using 25 mM phosphate buffer as an eluent. The fractions were tested for sucrose synthetase activity, pooled, and concentrated using an Amicon PM-30 ultrafiltration membrane. The enzyme was stored frozen at -20 °C.

Assay of Sucrose Synthetase. Activity measurements of the forward reaction were measured by monitoring the formation of UDP at various times with HPLC. Each 1.0 mL reaction vessel contained 50 mM HEPES buffer pH 7.5, 1.0 mM UDPG, 10 mM fructose and an appropriate amount of sucrose synthetase. The UDP-glc and UDP were separated on a Whatman SAX anion exchange HPLC column using 125 mM KH_2PO_4 as an eluent. The standard assay for the reverse reaction mixture contained 1.0 mM UDP, 10 mM sucrose, and 50 mM HEPES buffer, pH 7.5, in a volume of 1.0 mL. A unit of sucrose synthetase activity is defined as the amount of enzyme needed to catalyze the formation of 1.0 μmole/hour of UDP at 25 °C from UDP-glc and fructose at pH 7.5.

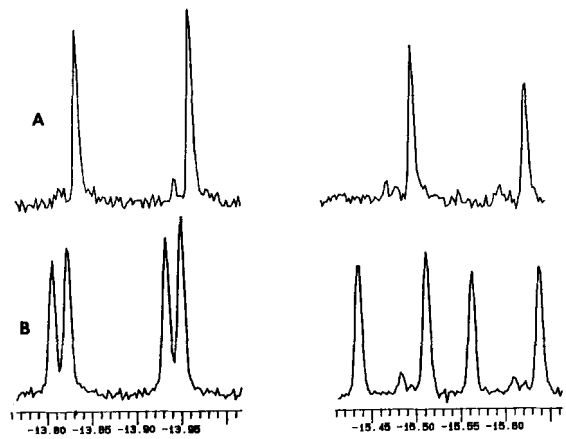


Figure 1: (A) ^{31}P NMR spectrum of [β-$^{18}O_2$,$\alpha\beta$-^{18}O]UDP-glc (III). The doublet for the α-P is centered at approximately -13.9 ppm and the doublet for the β-P is centered at approximately -15.55 ppm. (B) ^{31}P NMR spectrum for a mixture of unlabelled UDP-glc and [β-$^{18}O_2$,$\alpha\beta$-^{18}O]UDP-glc (III).

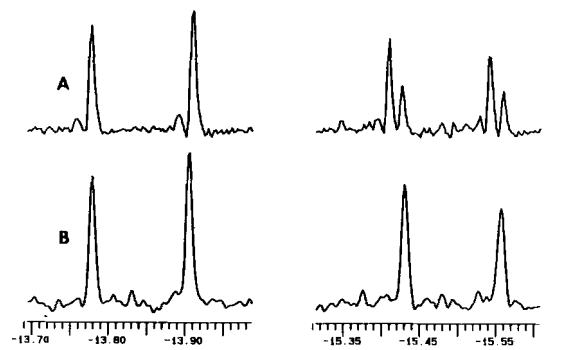


Figure 2: (A) ^{31}P NMR spectrum for [β-$^{18}O_2$,$\alpha\beta$-^{18}O]UDP-glc after incubation with fructose and sucrose synthetase. (B) ^{31}P NMR spectrum for [β-$^{18}O_2$,$\alpha\beta$-^{18}O]UDP-glc after incubation with sucrose synthetase. Additional details are given in the text.