Galactose-1-phosphate Uridylyltransferase. Purification of the Enzyme and Stereochemical Course of Each Step of the Double-Displacement Mechanism†

A. Arabshahi, R. S. Brody,‡ A. Smallwood, T.-C. Tsai,§ and P. A. Frey*

Institute for Enzyme Research, The Graduate School, and Department of Biochemistry, College of Agricultural and Life Sciences, University of Wisconsin, Madison, Wisconsin 53705, and Department of Chemistry, The Ohio State University, Columbus, Ohio 43210

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ABSTRACT: A convenient new procedure for purifying galactose-1-phosphate uridylyltransferase from *Escherichia coli* is described. It departs from earlier methods by introducing the use of a Cibacron Blue–agarose (Bio-Rad Affi-Gel Blue) at an early stage. Purification is completed by ion-exchange chromatography using DEAE-Sephadex A-50. The procedure is substantially shorter than earlier methods and reproducibly yields enzyme of high specific activity suitable for use in structural work such as characterizaton of the intermediate uridylyl-enzyme. The first step of the galactose-1-P uridylyltransferase reaction is the transfer of the uridylyl group from UDP-glucose to N^3 of a hisitidine residue in the enzyme to form the covalent uridylyl-enzyme and glucose-1-P. The uridylyl-enzyme intermediate then reacts in a second step with galactose-1-P to form UDP-galactose. The enzyme accepts (R_P)-UDPαS-glucose as a good substrate, converting it to (R_P)-UDPαS-galactose, i.e., with overall retention of configuration. In this paper we show that reaction of the enzyme with (R_P)-[2-^{14}C]UDPαS-glucose produces a [2-^{14}C]uridylylαS-enzyme that can be converted by base-catalyzed cyclization to (R_P)-[2-^{14}C]cUMPS. Inasmuch as cyclization must have proceeded with inversion of configuration at phosphorus, the corresponding configuration in the intermediate must have been the inverse of that in the substrate. Therefore, formation of uridylylαS-enzyme from (R_P)-UDPαS-glucose proceeds with *inversion* of configuration, and overall retention arises from inversion in each of the two steps. The results support the authenticity of the isolated uridylyl-enzyme as the true reaction intermediate. This is the first example of a double-displacement enzymatic reaction involving a covalent phosphoenzyme in which the stereochemical course of each step of the mechanism has been established.

Galactose-1-phosphate uridylyltransferase catalyzes the interconversion of UDP-glucose and galactose-1-P with UDP-galactose and glucose-1-P by a double-displacement mechanism involving the formation of a covalent uridylyl-enzyme as a compulsory intermediate according to eq 1 and 2. Evidence for this mechanism is outlined in the review by Frey et al. (1982) for the enzyme from *Escherichia coli*.

$$\text{E–His} + \text{UDP-glucose} \rightleftharpoons \text{glucose-1-P} + \text{E–His–}N^3\text{-UMP} \quad (1)$$

$$\text{E–His–}N^3\text{-UMP} + \text{galactose-1-P} \rightleftharpoons \text{E–His} + \text{UDP-galactose} \quad (2)$$

An important part of the evidence supporting the double-displacement mechanism was stereochemical, in that it was shown by using a chiral phosphorothioate analogue of UDP-glucose as substrate that the reaction proceeds with overall retention of configuration at phosphorus. Thus, uridine 5′-(1-thiodiphosphate) glucose [(R_P)-UDPαS-glucose][1] was converted to (R_P)-UDPαS-galactose; the reaction proceeded at about 7% of the rate at which UDP-glucose itself would have reacted. This finding supported the proposed mechanism because the mechanisms of eq 1 and 2 are essentially the microscopic reverse of each other. Therefore, whatever stereochemical consequences were involved in forming the uridylylαS-enzyme (E–His–N^3-UMPS) from R_P-UDPαS-glucose by eq 1, they had to be reversed upon reaction of the intermediate with galactose-1-P in eq 2 to form (R_P)-UDPαS-galactose. Thus, the mechanism required overall stereochemical retention; this was confirmed.

The stereochemical course of *each step* of the mechanism has not, until now, been established. Nor has it been for any other two-step nucleotidyltransferase or phosphotransferase. Overall configurational retention would have resulted either from inversion or retention of configuration in each step. In this paper we show that each step of the galactose-1-P uridylyltransferase reaction proceeds with *inversion* of configuration at phosphorus. We also describe a new, much shorter

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*Author to whom correspondence should be addressed at the Institute for Enzyme Research, University of Wisconsin.

‡Present address: Battelle Memorial Institute, Columbus, OH 43210.

§Present address: Providence College, Taichung, Taiwan.

[1] Abbreviations: (R_P)-UDPαS-glucose, uridine 5′-(1-thiodiphosphate) glucose having the *R* configuration at P[1]; (R_P)-UDPαS-galactose, uridine 5′-(1-thiodiphosphate) galactose having the *R* configuration at P[1]; UDP-glucose, uridine 5′-diphosphate glucose; UDP-galactose, uridine 5′-diphosphate galactose; UMP, uridine 5′-phosphate; UMPS, uridine 5′-phosphorothioate; (R_P)- and (S_P)-*p*-nitrophenyl-UMPS, *O*-(5′-uridine) *O*-(4-nitrophenyl) phosphorothioates having the *R* and *S* configurations at phosphorus; (R_P)- and (S_P)-imidazolyl-UMPS, *O*-5′-uridine *N*-imidazolylphosphorothioamidates having the *R* or *S* configuration at phosphorus; (R_P)- and (S_P)-cUMPS, uridine 3′,5′-cyclic phosphorothioates having the *R* and *S* configuration at phosphorus; (R_P)- and (S_P)-cAMPS, adenosine 3′,5′-cyclic phosphorothioates having the *R* and *S* configurations at phosphorus; PMSF, phenylmethanesulfonyl fluoride; NADPH, reduced nicotinamide adenine dinucleotide phosphate; NMR, nuclear magnetic resonance; HPLC, high-performance liquid chromatography; EDTA, ethylenediaminetetraacetic acid; TLC, thin-layer chromatography; cUMP, uridine 3′,5′-cyclic phosphate.

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and improved purification procedure for this unstable enzyme.

Experimental Procedures

Materials. Triimidazolyl phosphorothioate was prepared by the procedure of Eckstein (1970), 2′,3′-*O*-(methoxymethylidene)uridine by the procedure of Griffin et al. (1967), and *O,O*-bis(*p*-nitrophenyl) phosphorochloridothioate by the procedure of Eckstein et al. (1974). Trimethyl orthoformate and dimethyl sulfoxide were redistilled from CaH and stored over molecular sieves (Linde 4a). Other solvents used in synthetic procedures were purified as described earlier (Brody & Frey, 1981). Potassium *tert*-butoxide was prepared as a 1 M solution by dissolving the appropriate weight of potassium metal in 2-methyl-2-propanol that had just been distilled from CaH. Aliquots of potassium *tert*-butoxide were sealed in glass tubes and stored at −20 °C. A tube was opened for use in each experiment. Affi-Gel Blue was purchased from Bio-Rad Laboratories. Sephadex gel filtration and ion-exchange media were purchased from Sigma Chemical Co. All other chemicals, buffer salts, coenzymes, substrates, and enzymes used for assaying galactose-1-P uridylyltransferase were purchased from commercial suppliers and used as supplied.

General Methods. Galactose-1-P uridylyltransferase was assayed in the direction of glucose-1-P production by coupling the reaction to NADPH formation by using phosphoglucomutase, glucose-6-P dehydrogenase, and necessary substrates and cofactors as described by Wong and Frey (1974). Thin-layer chromatography was carried out by using Eastman silica gel plates containing a fluorescent indicator. Ultraviolet absorbance measurements were recorded by using a Pye-Unicam SP 1800 spectrophotometer. ^{1}H NMR spectra were obtained on a Bruker WP-200 spectrometer operating at 200 MHz. Peak positions were referenced to the trimethylsilyl peak of sodium 3-(trimethylsilyl)[2,2,3,3-$^2H_4$]propionate (δ 0.00; Merck). ^{31}P NMR spectra were obtained on the same spectrometer operating at 81 MHz. The spectra were referenced to H_3PO_4 as an external standard (δ 0.00) and were proton spin decoupled unless otherwise stated. Radiochemical assays were carried out by liquid scintillation counting on a Beckman LS 100C spectrometer. High-performance liquid chromatography was carried out on a Waters Model 440 chromatograph equipped with ultraviolet and refractive index detectors. Reverse-phase C_{18} Bondapak columns (semipreparative, 0.39 × 30 cm) were used, with either water or phosphate buffer as the mobile phase.

Purification of Gal-1-P Uridylyltransferase. Escherichia coli cells (ATCC 27797) were grown as described earlier (Wee & Frey, 1973), frozen as pellets in liquid N_2, and stored at −70 °C. All purification procedures were carried out at 0–5 °C, and all buffers contained 1 mM EDTA, 1 mM 2-mercaptoethanol, and 1 mM PMSF. This level of PMSF was added daily to all buffers. All centrifugations were at 14000*g* for 40 min. Frozen cells, 70 g, were suspended in 250 mL of 10 mM KP_i buffer at pH 7.5 by homogenizing the mixture for about 1 min in a blender. The cooled cells were broken by sonication in four 1-min bursts with a Branson sonifier, the temperature being maintained below 5 °C by cooling the suspension between bursts. Cell debris was removed by centrifugation. The supernatant fluid was brought to 3% streptomycin sulfate by addition of a 20% solution at pH 7.5 and then stirred for 20 min. The precipitate was removed by centrifugation and the supernatant fluid adjusted to pH 8.0 by addition of 1 M NaOH. Solid $(NH_2)_2SO_4$ was added to 35% saturation and the solution stirred for 20 min in an ice–water bath. After the precipitate was removed by centrifugation, additional $(NH_4)_2SO_4$ was added to 70% saturation. After the solution was stirred for 20 min, the precipitated enzyme was harvested by centrifugation, dissolved in about 100 mL of 2 mM sodium bicinate at pH 8.5, and desalted by passage through a 1-L column of Sephadex G-25, equilibrated with the same buffer. Active fractions were pooled and diluted to 4 mg of protein·mL^{-1}, and the solution was passed through a 4 × 25 cm column of Affi-Gel Blue at the maximum flow rate. The column was eluted with a 1.5-L linear gradient of NaCl increasing from 0 to 0.5 M prepared in 2 mM sodium bicinate at pH 8.5. Fractions with enzyme activity of 25 units·mg^{-1} or higher were pooled and desalted by passage in two portions through the above Sephadex G-25 column, equilibrated, and eluted with 5 mM KP_i buffer at pH 7.5. The desalted enzyme was loaded onto a 2 × 30 cm column of DEAE-Sephadex A-50 in the $HOPO_3^{2-}$ form. The column was eluted with a 1-L linear gradient of KP_i buffer at pH 7.5 increasing in concentration from 5 to 86 mM. Fractions having specific activities greater than 180 units·(mg of protein)$^{-1}$ were pooled and desalted by passage through the above described Sephadex G-25 column. To concentrate the enzyme, the solution was passed through a 0.5-mL column of DEAE-Sephadex A-50 in the $HOPO_3^{2-}$ form and the absorbed enzyme eluted with 0.1 M KP_i at pH 7.5.

Synthesis of Nucleotides. The epimer mixture (R_P + S_P)-*p*-nitrophenyl-UMPS was synthesized essentially as described by Burgers et al. (1979) for the adenine nucleotide. 2′,3′-(Methoxymethylidene)uridine-5′ bis-(*p*-nitrophenyl) phosphorothioate was produced by reaction of *O,O*-bis-(*p*-nitrophenyl) phosphorochloridothioate (1.21 g, 3.2 mmol) with 2′,3′-(methoxymethylidene)uridine (0.7 g, 2.7 mmol) as described by Burgers et al. (1979). The solid product was dissolved in 60 mL of dioxane (passed over neutral alumina to remove peroxides), and 60 mL of 0.2 M NaOH was added with stirring. Analysis by thin-layer chromatography (silica gel; chrloroform/CH_3OH, 92:8) showed that no starting material remained after 1 h. The pH was adjusted to 5 with dilute H_2SO_4, and the solution was brought to dryness by rotary evaporation. Ethanol added to the residue was removed by rotary evaporation. The product was dissolved in methanol and filtered to remove Na_2SO_4. The filtrate was concentrated to dryness by rotary evaporation, the residue dissolved in 100 mL of 1:1 v/v dioxane/water, and the pH adjusted to 2 with 1 mM HCl and held at 25 °C for 2 h. *p*-Nitrophenol was removed by extraction with three 50-mL portions of $CHCl_3$ and the aqueous layer concentrated to dryness by rotary evaporation. The yield was 2.4 mmol (88%). It migrated as a single UV quenching spot by thin-layer chromatography (silica gel; 1-propanol/concentrated NH_3/H_2O, 6:3:1) with an R_f of 0.56. The ultraviolet spectrum consisted of a maximum at 266 nm and shoulder at 294 nm (A_{266}/A_{294} = 1.7). Proton spin decoupled ^{31}P NMR (D_2O/H_2O, 2:1, pH 8.5) showed two peaks of equal intensity for the two epimers at δ 52.20 and 52.04 relative to an external reference of H_3PO_4.

The epimers of *p*-nitrophenyl-UMPS were separated by reverse-phase HPLC using a Waters semipreparative C_{18} Bondapak column with H_2O as the mobile phase. Samples of 35 μmol in 0.25 mL were applied to the column at a flow rate of 2 mL·min^{-1}, and the two epimers (15 μmol of each) emerged at 80 (R_P) and 110 m (S_P). Configurational assignment was by sensitivity to snake venom phosphodiesterase, which is specific for the R_P epimer (Burgers et al., 1979). The epimers, separately collected and concentrated by rotary evaporation, gave the following NMR data: for (R_P)-*p*-nitrophenyl-UMPS, ^{31}P NMR δ 52.17 (s), ^{1}H NMR δ 8.243 (d, J = 9 Hz, 2 H, *p*-nitrophenyl), 7.849 (d, J = 8 Hz, 1 H,

H_6), 7.397 (d, J = 9 Hz, 2 H, p-nitrophenyl), 5.952 (d, J = 4 Hz, 1 H, $H_{1'}$), 5.731 (d, J = 8 Hz, 1 H, H_5); for (S_P)-p-nitrophenyl-UMPS, ^{31}P NMR δ 52.04 (s), ^{1}H NMR δ 8.245 (J = 9 Hz, 2 H, p-nitrophenyl), 7.799 (d, J = 8 Hz, 1 H, H_6), 7.387 (doublet of doublets, J = 9, 1 Hz, 2 H, p-nitrophenyl), 5.937 (d, J = 4 Hz, 1 H, $H_{1'}$), 5.698 (d, J = 8 Hz, 1 H H_5).

Imidazolyl-UMPS was synthesized as a mixture of epimers by a procedure based on that described by Eckstein (1970). Triimidazolyl phosphorothioate (2.65 g, 10 mmol, dried at 100 °C, 30 min, 0.1 Torr) was added to a solution of uridine (2.44 g, 10 mmol, dried at 100 °C, 30 min, 0.1 Torr) in 40 mL of dry pyridine, and the solution was stirred under N_2 for 5 h. After filtration of the precipitate, the filtrate was combined with 40 mL of H_2O and concentrated to dryness by rotary evaporation. The residue was redissolved in H_2O, the pH adjusted to 9 by addition of 0.5 M NaOH, and the solution applied to a column of DEAE-Sephadex (bed volumne 250 mL) in the HCO_3^- form. The column was eluted at 4 °C with a linear gradient of triethylammonium bicarbonate increasing from 0.05 to 0.2 M (total volume 5 L) at pH 9.0. The product emerged at 0.12 M salt, and pooled fractions were dried by rotary evaporation. Ethanol was added and the product again dried. Imidazolyl-UMPS migrated as a single spot by TLC (silica gel; 1-propanol/concentrated NH_3/H_2O, 6:3:1, R_f 0.5) with faint impurity spots of lower R_f values. The yield was 2.7 mmol (27%). The compound decomposed slowly at −20 °C and so was stored at −60 °C at pH 8.0. After conversion to the Na^+ form (AG-50, Na^+), the ^{1}H and ^{31}P NMR assignments were as follows: ^{1}H NMR, δ 8.17 (s, 1 H, imidazolyl), 7.78 (t, 1 H, H_6), 7.42 (s, 1 H, imidazolyl), 7.15 (s, 1 H, imidazolyl), 5.90 (m, 2 H, H_5 + $H_{1'}$). The imidazolyl signals were broadened relative to the uracil signals: ^{31}P NMR δ 47.31 (s), 47.20 (s).

The epimers of imidazolyl-UMPS were separated by HPLC using a reverse-phase Waters semipreparative C_{18} Bondapak column and 5 mM potassium phosphate (pH 7.5) as the eluent. At a flow rate of 2 mL·min^{-1} one epimer emerged at 110 min and the second at 155 min. The pure epimers were relatively stable in dilute solutions but underwent slow epimerization to the mixture in concentrated solutions at pHs 4.2 and 7.5, as revealed by ^{31}P NMR analysis of samples that had been rotary evaporated to dryness and then redissolved. A sample at pH 9.2 similarly evaporated and redissolved showed no loss of epimeric purity.

[2-^{14}C]UDPαS-glucose was synthesized as a mixture of epimers (R_P + S_P) from [2-^{14}C]UMPS and glucose-1-P by a minor modification of the procedure described by Sheu et al. (1979) using [2-^{14}C]UMPS prepared from [2-^{14}C]uridine and $PSCl_3$. The K^+ salts of the epimers were separated by reverse-phase HPLC using a semipreparative C_{18} Bondapak column and 50 mM potassium phosphate at pH 6.0 as the mobile phase. The flow rate was 2 mL·min^{-1}, and retention times were 9.8 min for epimer S_P and 14.6 min for epimer R_P. The pure epimers were collected and desalted by absorption to DEAE-Sephadex columns, elution with triethylammonium bicarbonate, and evaporation of the volatile buffer by rotary evaporation. The purity of the epimers was verified by HPLC and ^{31}P NMR analysis; the ^{31}P NMR data were the same as those reported earlier for the same compounds prepared enzymatically from the mixture of (R_P)-UDPαS-glucose and (S_P)-UDPαS-glucose (Sheu et al., 1979). The specific radioactivity of [2-^{14}C]UDPαS-glucose was 9.33 × 10^4 cpm·μmol^{-1}.

Cyclizations of p-Nitrophenyl-UMPS and Imidazolyl-UMPS. Samples of (R_P + S_P)-p-nitrophenyl-UMPS (40 μmol), (R_P)-p-nitrophenyl-UMPS (26 μmol), (S_P)-p-nitrophenyl-UMPS (25 μmol), and (R_P + S_P)-imidazolyl-UMPS (40 μmol) were dried at 25 °C under vacuum overnight and further dried for 20 min at 56 °C and 0.1 Torr. The samples were dissolved in 2.25-mL portions of dimethyl sulfoxide, and 0.25-mL aliquots of 1 M potassium *tert*-butoxide were added to each sample. After 20 min at room temperature excess cation-exchange resin (AG-50, NH_4^+) and 5 mL of H_2O were added to the reaction mixtures. The suspensions were filtered to remove resins, which were washed with dilute NH_3 (pH 10). The water was removed from each sample by rotary evaporation and the remaining dimethyl sulfoxide removed under high vacuum at 50 °C by using a short path distillation apparatus. The cyclization products were analyzed by HPLC, ^{31}P NMR and ^{1}H NMR, which showed that in all cases cyclization produced (R_P)- or (S_P)-cUMPS or both, depending upon which substrate was used. The following NMR data were obtained: for (R_P)-cUMPS, ^{31}P NMR δ 55.75 (s), ^{1}H NMR δ 7.696 (d, J = 8 Hz, 1 H, H_6), 5.892 (d, J = 8 Hz, 1 H, H_5), 5.829 (s, H_1); for (S_P)-cUMPS, ^{31}P NMR δ 54.12 (s), ^{1}H NMR δ 7.662 (d, J = 8 Hz, H_6), 6.001 (d, J = 8 Hz, H_5), 5.816 (s, $H_{1'}$). (R_P)- and (S_P)-cUMPS were separated by HPLC using a Waters semipreparative C_{18} Bondapak column and as the mobile phase 5 mM KH_2PO_4. At a flow rate of 2 mL·min^{-1} the retention times for (R_P)- and (S_P)-cUMPS were 7.2 and 9.0 min, respectively. An authentic sample of (S_P)-cUMPS prepared as described by Baraniak et al. (1982) and kindly supplied by W. J. Stec migrated precisely with the product resulting from cyclization of (R_P)-p-nitrophenyl-UMPS.

Cyclization of the [2-^{14}C]UridylylαS Moiety of [2-^{14}C]-UridylylαS-galactose-1-P Uridylyltransferase to (R_P)-[2-^{14}C]cUMPS. Galactose-1-P uridylyltransferase, 2 mg purified as described above, was incubated with 5 μmol of (R_P)-[2-^{14}C]UDPαS-glucose in 1 mL of 0.1 M potassium phosphate buffer at pH 7.5. After 5 min at 25 °C the solution was chilled in an ice–water bath and treated with 20 μL of acetic anhydride. The pH was maintained between 7 and 10 by microliter additions of 1 M KOH until it became stable. The acetic anhydride addition was repeated four additional times at 0 °C, the pH being maintained each time between 7 and 10 by additions of base. The solution was then passed through a 2.0 × 25 cm column of Sephadex G-25 equilibrated and eluted with water adjusted to pH 10 with KOH. Fractions of 1 mL volume were collected and assayed for protein (A_{280}) and ^{14}C. The radioactive protein-containing fractions were pooled and stored at −20 °C. The [^{14}C]uridylylαS proteins from two experiments were combined (3 mg, 8100 cpm of ^{14}C), concentrated by rotary evaporation, and lyophilized. The [^{14}C]uridylylαS protein was further dried in a vacuum desiccator over P_2O_5 for 48 h at ambient temperature. The dried residue was dissolved in 10 mL of dry dimethyl sulfoxide and 1.5 mL of potassium *tert*-butoxide solution (1 M) added. The reaction mixture was magnetically stirred for 20 min at ambient temperature and then neutralized by addition of 3 mL of AG-50 ion-exchange resin in the triethylammonium form to stop the reaction. After the mixture was filtered through glass wool, the filtrate was diluted to 100 mL with water and applied to a 1.5 × 13 cm column of DEAE-Sephadex A-25 in the bicarbonate form. The column was washed with 100 mL of 0.025 M triethylammonium bicarbonate and then eluted with 0.3 M triethylammonium bicarbonate. Radioactive fractions (1875 cpm) were pooled, concentrated to dryness by rotary evaporation combined with carrier cUMPS (approximately 1 μmol of R_P and 0.1 μmol of S_P) and rechromato-

Table I: Purification of Galactose-1-P Uridylyltransferase[a]

step	protein (mg)	enzyme activity (units)	specific activity (units·mg^{-1})	yield (%)
extract	7020	2000	0.30	
streptomycin sulfate	5800	2000	0.35	100
$(NH_4)_2SO_4$ fraction	3190	1650	0.50	83
Sephadex G-25	2736	1600	0.55	80
Affi-Gel Blue	40	1220	30	60
DEAE-Sephadex I	3	657	219	33
DEAE-Sephadex II	1.9	448	238	22

[a] The detailed procedure is described under Experimental Procedures.

graphed as described above, yielding 19 A_{260} units of nucleotide containing 1675 cpm of ^{14}C. The radioactive fraction was again concentrated to dryness as above, dissolved in 3 mL of H_2O, and filtered through a Millipore membrane filter (Millex-GS, 0.22 μm). Samples of the filtrate up to 750 μL were subjected to HPLC through a semipreparative Waters C_{18} Bondapak reverse-phase column using 6 mM KH_2PO_4 as the mobile phase at a flow rate of 1 mL·min^{-1} and 900 psi. The effluent was collected in 1-min fractions, which were analyzed for nucleotide (A_{260}) and radioactivity. (R_P)-cUMPS and (S_P)-cUMPS were injected just prior to the injection of ^{14}C-labeled nucleotide and emerged from the column with retention times of 12 and 15 min, respectively.

Conversion of (R_P)-[2-^{14}C]cUMPS to [2-^{14}C]cUMP. Four samples of galactose 1-P uridylyltransferase (7 mg total) were converted to [2-^{14}C]uridylylαS-enzyme, pooled, and cyclized to (R_P)-[2-^{4}C]cUMPS as described. The (R_P)-[2-^{14}C]cUMPS was isolated by DEAE-Sephadex chromatography and HPLC as for [2-^{14}C]cUMPS described above. Alternate HPLC fractions were used for detecting (R_P)-[2-^{14}C]cUMPS; the remaining HPLC fractions containing the enzyme-derived ^{14}C-labeled compound were concentrated by rotary evaporation to 0.20 mL and treated with 0.14 mL of 0.17 M KIO_3 dissolved in 0.1 M potassium phosphate buffer at pH 4. After 12 h at 25 °C the solution was filtered through a 0.45-μm Millex-HV filter and washed through with 0.4 mL of water in preparation for HPLC. The effluent A_{260} was continuously monitored and recorded with the UV-detection system linked to the chromatograph. Effluent was collected in 1-mL fractions which were subjected to radiochemical analysis.

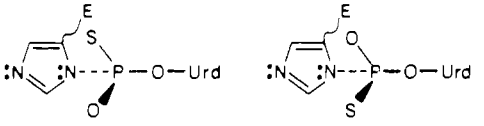
Results

Purification of Galactose-1-P Uridylyltransferase. To trace the stereochemistry of both steps in the galactose-1-P uridylyltransferase reaction, it was necessary to prepare the enzyme reproducibly in milligram quantities. The published purification procedure (Saito et al., 1967) has been used, with modifications, for earlier work that did not require large amounts of enzyme. Because of the extreme lability of this enzyme to proteolysis and oxidation, this rather lengthy purification proved to be inconvenient for the present purposes. Therefore, we devised a much shorter procedure that reproducibly yields enzyme of high purity.

The purification of galactose-1-P uridylyltransferase is outlined in Table I together with typical data. The enzyme is protected from proteolysis and oxidation during purification by daily additions of PMSF, EDTA, and 2-mercaptoethanol to all buffers. After nucleic acids are removed by streptomycin sulfate precipitation and a salt fractionation is carried out to remove some proteins and various other soluble contaminants in the extract, the enzyme is desalted by passage through a large column of Sephadex G-25. It is then immediately passed through a large column of Bio-Rad Affi-Gel Blue. Most proteins pass through this column, but galactose-1-P uridylyltransferase is retained. Elution of the column with a salt gradient further separates this enzyme from other proteins initially retained with it and results in a 55-fold purification from the preceding step. After desalting, again by passage through a large column of Sephadex G-25, the enzyme is chromatographed through a column of DEAE-Sephadex A-50, which affords an additional 6-fold purification to near homogeneity. Rechromatography through a small DEAE-Sephadex A-50 column both concentrates the enzyme and further purifies it to over 200 units·(mg of protein)$^{-1}$, the highest specific activity so far reported.

When purified as described above with no delays between steps, the enzyme can routinely be obtained in a nearly homogeneous form having a high specific activity. Relatively stable in extracts, it is increasingly labile to proteolysis and oxidation as it is purified and so must be carefully protected during purification and used promptly to prepare uridylyl-enzyme or uridylylαS-enzyme. The enzyme has a tendency to undergo transition to a lower specific activity form (approximately 50 units·mg^{-1}) that avidly binds UDP-glucose as an intact entity. This form of the enzyme can be converted to the uridylyl-enzyme in only low yield, so it is important in preparing the free uridylyl-enzyme to use freshly purified enzyme.

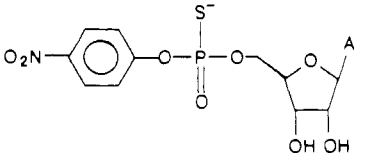
Cyclization of p-Nitrophenyl-UMPS and Imidazolyl-UMPS. In the covalent uridylyl-enzyme intermediate the uridylyl group is bonded to N3 of a histidyl residue (Yang & Frey, 1979). In the intermediate produced by reaction of (R_P)-UDPαS-glucose, the uridylylαS group must have a chiral P with either the (*S*)-**1a** or (*R*)-**1b** configuration. The con-



1a **1b**

figuration in the intermediate can be established if the uridylylαS moiety can be cyclized to uridine 3′,5′-cyclic phosphorothioate (cUMPS) by a route that follows a known stereochemical course. The product configuration can be assigned by comparison with authentic samples of (R_P)-cUMPS and (S_P)-cUMPS and the intermediate configuration deduced from the known stereochemistry of the cyclization reaction.

Cyclizations of 5′-*O*-nucleoside *N*-imidazolyl phosphorothioamidates are unknown. However, cyclizations of *p*-nitrophenyl-AMPS (**2**) to cAMPS and P^1-(3′-uridine) P^2-diethyl 1-thiodiphosphate (**3**) to uridine 2′,3′-*O*,*O*-cyclic



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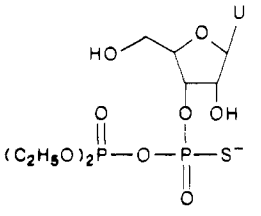

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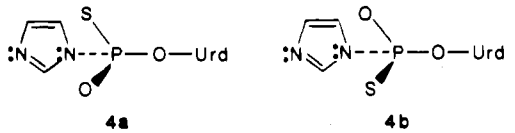
phosphorothioate, as well as other P^1-(2-hydroxalkyl) P^2-diphenyl 1-thiodiphosphates to corresponding cyclic phosphorothioates, are catalyzed by potassium *tert*-butoxide in polar, nonaqueous solvents (Burgers et al., 1979; Usher et al., 1970; Orr et al., 1978). These reactions proceed with inversion of configuration at phosphorus.

To determine the feasibility of cyclizing **1a** or **1b** to cUMPS, *N*-imidazolylphosphorothioamidates **4a** and **4b**, (S_P)- and



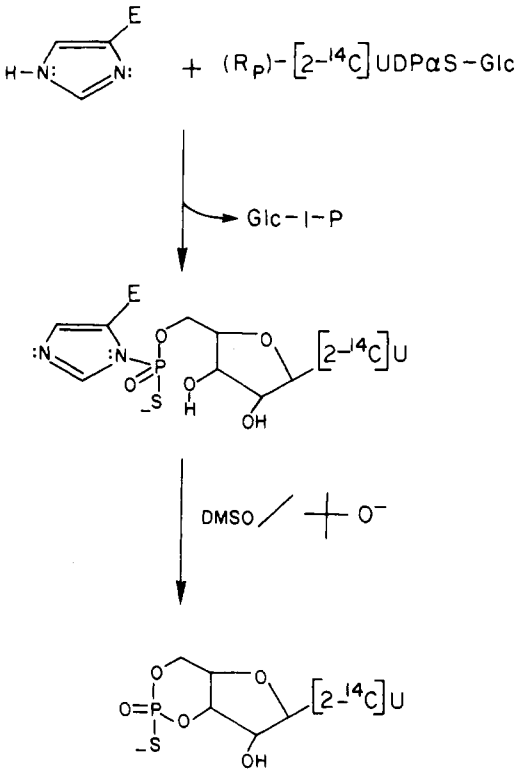
4a **4b**

(R_P)-imidazolyl-UMPS,[2] were synthesized as a mixture of epimers and some of their properties investigated. (R_P)-cUMPS and (S_P)-cUMPS were also synthesized by cyclizing (S_P)-*p*-nitrophenyl-UMPS and (R_P)-*p*-nitrophenyl-UMPS, respectively (see Experimental Procedures). Cyclizations of **4a** and **4b** to (S_P)-cUMPS and (R_P)-cUMPS proceeded in dimethyl sulfoxide at 25 °C within 30 min in the presence of potassium *tert*-butoxide. The cyclization experiments were carried out with the mixture of **4a** and **4b**, and yields were estimated by ^{31}P NMR and HPLC. The maximum yields were about 50% for the production of (S_P)-cUMPS and 20% for (R_P)-cUMPS on the basis of the ratio of (R_P)- and (S_P)-imidazolyl-UMPS in the mixture and the overall recovery of (S_P)- and (R_P)-cUMPS. The cyclization conditions were the same as those used to produce (S_P)-cUMPS and (R_P)-cUMPS from (R_P)-*p*-nitrophenyl-UMPS and (S_P)-*p*-nitrophenyl-UMPS. (R_P)-*p*-Nitrophenyl-UMPS was found to cyclize in higher yield than (S_P)-*p*-nitrophenyl-UMPS and with the formation of fewer and smaller amounts of side products. This was also reported for similar cyclizations of (R_P)- and (S_P)-*p*-nitrophenyl-AMPS (Burgers et al., 1979).

The epimers **4a** and **4b** were well separated by HPLC and could be isolated. Although somewhat unstable when stored at −20 °C, they could be stored at −60 °C at pHs above 8. At pHs below 7 pure **4a** or **4b** underwent epimerization to the mixture **4a** + **4b**, as well as significant hydrolysis to UMPS, when solutions were evaporated to dryness and the samples redissolved. The compounds remained stereochemically pure for hours in dilute aqueous solutions at pHs as low as 4.2 and showed no tendency to undergo epimerization or hydrolysis at pH 9, even when evaporated to dryness and redissolved. These properties were compatible with the hydrolytic properties reported for uridylyl–galactose-1-P uridylyltransferase (Wong et al., 1977).

Cyclization of [2-^{14}C]UridylylαS-enzyme to (R_P)-[2-^{14}C]cUMPS. To investigate the effects of the presence of protein and small amounts of water on cyclizations of uridylylαS-enzyme, we studied the cyclization of (R_P + S_P)-*p*-nitrophenyl-UMPS in the presence of added H_2O or bovine serum albumin. The cyclization solutions contained 2.25 mL of dimethyl sulfoxide, 20 μmol of (R_P + S_P)-*p*-nitrophenyl-UMPS and 0.25 mL of 1 M potassium *tert*-butoxide. In the standard reaction the yield of (R_P)-cUMPS was 60% that of (S_P)-cUMPS. In the presence of 20–55 μmol of added water the yield of (R_P)-cUMPS decreased by 45% while that of (S_P)-cUMPS decreased by 30%. In the presence of

Scheme I



10 mg of bovine serum albumin the yield of (R_P)-cUMPS decreased by 80% whereas that of (S_P)-cUMPS decreased by 54%. The results indicated that lower yields of cUMPS could be expected from uridylylαS-enzyme than from model compounds **4a** and **4b** and a substantially lower yield from the R_P epimer (**1b**).

Scheme I outlines the procedure for preparing [2-^{14}C]-uridylylαS-enzyme from (R_P)-[2-^{14}C]UDPαS-glucose and galactose-1-P uridylyltransferase and cyclizing the [2-^{14}C]-uridylylαS group to [2-^{14}C]cUMPS. Efficient uridylylation of the enzyme at 2 mg·mL^{-1} proceeds with 4 mM (R_P)-[2-^{14}C]UDPαS-glucose, consistent with the K_{eq} for reaction with UDP-glucose (Wong & Frey, 1974). Immediately after uridylylation the enzyme is exhaustively acetylated by reaction with acetic anhydride, which acetylates the primary amino groups and enhances the solubility of the protein in the cyclizing solvent dimethyl sulfoxide. The radioactive protein is then isolated from unreacted [2-^{14}C]UDPαS-glucose by gel filtration and lyophilized. The residue is further dried in a vacuum desiccator and dissolved in dimethyl sulfoxide, and potassium *tert*-butoxide is added.

After cyclization the ^{14}C-labeled nucleotides were isolated by chromatography through DEAE-Sephadex A-25 and prepared for reverse-phase HPLC under conditions suitable for separating (R_P)-cUMPS and (S_P)-cUMPS, whose retention times were measured in the same system immediately before chromatography of the ^{14}C-labeled nucleotide. The results in Figure 1 were obtained from a portion of the product derived from 3 mg of galactose-1-P uridylyltransferase taken through this procedure. The protein contained initially 8100 cpm of [2-^{14}C]uridylylαS groups (1.1 mol/mol of subunits), 5% of which were isolated with (R_P)-cUMPS from the HPLC column. On the basis of the yield of (R_P)-cUMPS from (R_P)-imidazolyl-UMPS and the effect of the presence of protein on the cyclization yield from (S_P)-*p*-nitrophenyl-UMPS, a 5% overall recovery was in the expected range for the S_P epimer of [2-^{14}C]uridylylαS-enzyme. The experiment

[2] For a configuration at P in imidazolyl-UMPS and *p*-nitrophenyl-UMPS, the opposite (*R*, *S*) stereochemical symbols apply. This results from application of the priority rules for assignment of symbols. It is also for this reason that the configurational symbols for (R_P)-UDPαS-Glc and (R_P)-uridylylαS-enzyme are the same, even though their configurations are opposite.

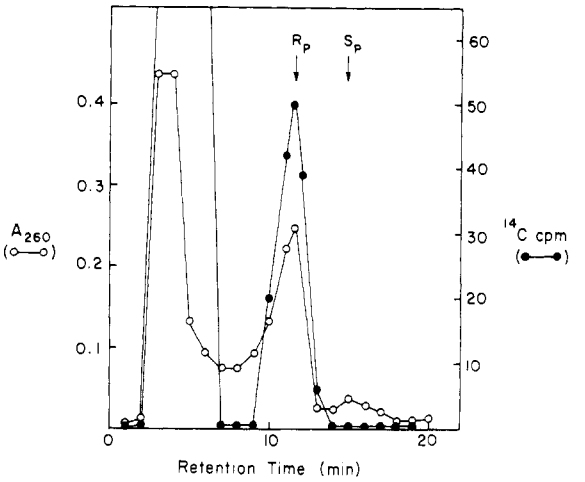
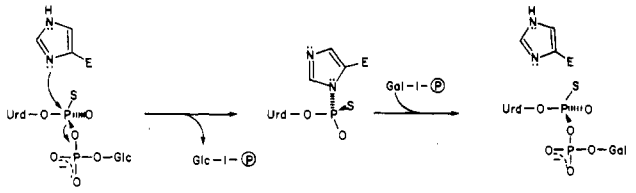


FIGURE 1: Isolation of (R_P)-[^{14}C]cUMPS from cyclization of [2-^{14}C]uridylylαS-enzyme. [2-^{14}C]UridylylαS–galactose-1-P uridylyltransferase was prepared, isolated, and subjected to cyclization conditions, and the uridine nucleotides were isolated as described under Experimental Procedures. The nucleotides were chromatographed through a Waters Bondapak C_{18} column (0.39 × 30 cm) equilibrated and eluted with 6 mM KH_2PO_4 at a flow rate of 1 mL/min and a pressure of 900 psi. Fractions were collected at 1-min intervals and analyzed for A_{260} and ^{14}C. Standard samples of (S_P)-cUMPS and (R_P)-cUMPS were chromatographed just before and just following the run illustrated in the figure and were eluted with retention times of 15.0 ± 0.5 and 12.0 ± 0.5 min, respectively, in close correspondence with the retention times of carrier in the experiment illustrated. (●) Radioactivity; (○) A_{260}.

Scheme II



has repeatedly produced comparable results, with product yields of 2–5%.

Figure 1 shows that cyclization of the [2-^{14}C]uridylylαS-enzyme produces a ^{14}C-labeled nucleotide that cochromatographs with (R_P)-cUMPS and *not* with (S_P)-cUMPS. To confirm that the ^{14}C-labeled nucleotide is (R_P)-cUMPS, a sample was prepared from 7 mg of enzyme and desulfurized by reaction with KIO_3 at pH 4, which converts cUMPS to cUMP. The product was then again subjected to analysis by HPLC under conditions in which cUMP, (R_P)-cUMPS, and (S_P)-cUMPS are well separated. The results shown in Figure 2 confirmed that the ^{14}C-labeled nucleotide resulting from the desulfurization process was [2-^{14}C]cUMP, which could only have been derived from (R_P)-[2-^{14}C]cUMPS.

We conclude that the configuration at phosphorus in [2-^{14}C]uridylylαS–galactose-1-P uridylyltransferase must be *R*, since ^{14}C is associated exclusively with (R_P)-cUMPS and not with (S_P)-cUMPS in Figure 1. This corresponds to structure **1b**, which is inverted relative to that of P_α in (R_P)-UDP S-Glc, and uridylylation of the enzyme must, therefore, proceed with *inversion* of configuration at phosphorus as illustrated in Scheme II. Inversion at each step of the reaction accounts for overall retention of configuration in the two steps of the galactose-1-P uridylyltransferase reaction.

DISCUSSION

Purification of Galactose-1-P Uridylyltransferase. Our purification procedure for galactose-1-P uridylyltransferase is convenient and reproducibly yields enzyme of high specific

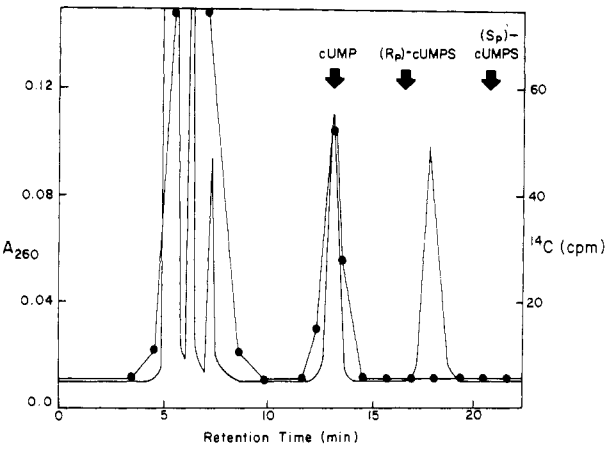


FIGURE 2: Conversion of (R_P)-[^{14}C]cUMPS to [^{14}C]cUMP. [2-^{14}C]UridylylαS–galactose-1-P uridylyltransferase was prepared and cyclized to (R_P)-cUMPS, and this was desulfurized by oxidation with KIO_3 as described under Experimental Procedures. The nucleotides were chromatographed through a Waters Bondapak C_{18} column (0.39 × 30 cm) equilibrated and eluted with 20 mM KH_2PO_4 in methanol/water (10:90) at a flow rate of 2 mL/min. The retention times of standard samples under these conditions were the following: KIO_3, 5.1 min; UDPαS-Glc, 6.5 min; cUMP, 13 min; (R_P)-cUMPS, 16.5 min; (S_P)-cUMPS, 20.5 min. (—) A_{260}; (●) radioactivity.

activity in acceptable yields. The principal advantage of this method is the early use of Affi-Gel Blue as a chromatography medium. This is a Cibacron Blue–agarose adsorbent that binds galactose-1-P uridylyltransferase and a few other proteins but does not retain most proteins in the extract. The enzyme, which can be eluted from Affi-Gel Blue with 5 mM ATP as well as salt, is not inhibited by this level of ATP. It is in these respects similar to phospholipase A_2, which also binds to Cibacron Blue but is not inhibited by ATP (Barden et al., 1980). The nature of the binding between the dye and this enzyme is unknown, but Affi-Gel Blue is a valuable chromatography medium.

Although Affi-Gel Blue is used in our procedure subsequent to streptomycin sulfate and ammonium sulfate fractionation, it can be used with the crude extract provided it is first passed through a column of Sephadex G-25. The Affi-Gel Blue column can be used repeatedly if it is thoroughly washed first with 6 M guanidinium chloride and then with H_2O after each use. However, the effectiveness of the column declines after six to eight weekly uses. The column must be thoroughly cleaned just before use; otherwise the salt elution releases blue dye from the agarose, and no enzyme activity can be recovered. It is also important to establish the enzyme-binding capacity of each newly prepared batch of Affi-Gel Blue before proceeding, since the capacity varies somewhat from batch to batch.

The enzyme is quite unstable and must be protected from oxidation and proteolysis. It loses activity relatively rapidly even when so protected; and added metal ions or/and substrates do not further protect it during prolonged storage. For this reason we use the enzyme immediately after purification.

Stereochemical Course of Uridylyl Group Transfer. Inasmuch as base-catalyzed cyclization of [2-^{14}C]uridylylαS-enzyme produces (R_P)-[2-^{14}C]cUMPS and *not* (S_P)-[2-^{14}C]-cUMPS, the configuration at phosphorus in the intermediate must be *R*, the inverse of that in the substrate (R_P)-[2-^{14}C]-UDPαS-Glc. Base-catalyzed cyclizations of this type are known to proceed with inversion of configuration (Westheimer, 1981). Therefore, the first step of the reaction mechanism, uridylyl group transfer from UPDαS-Glc to a histidine N3 of the enzyme, proceeds with *inversion* of configuration at

phosphorus, and the conversion of (R_P)-UDPαS-Glc to (R_P)-UDPαS-Gal proceeds with overall *retention* of configuration at phosphorus because each of the two steps in the double-displacement pathway (eq 1 and 2) proceeds with inversion. The galactose-1-P uridylyltransferase reaction is the first example of a double-displacement enzymatic reaction involving a covalent phosphoenzyme in which the stereochemical course of each step in the mechanism has been determined.

Our configurational assignment to [2-^{14}C]uridylylαS-enzyme is based on degradation of this intermediate to (R_P)-[2-^{14}C]cUMPS. This is a logical approach but leads in this case to only 2–5% yield of cyclic nucleotide. The low yield is accounted for by the fact that the configuration at phosphorus in the intermediate is *R*. This is because the yields in the cyclizations of the models (R_P)-imidazolyl-UMPS (**4b**) (20%) and (S_P)-*p*-nitrophenyl-UMPS (60%) are lower than those from the opposite epimers and also because the presence of protein in cyclizations has a much greater negative effect on product yield from one epimer than from the other. If the configuration in the intermediate had been *S*, the overall yield of cUMPS in Figure 1 would have been much higher, perhaps 20–25%. The configuration in (R_P)-uridylylαS-enzyme is of course governed by the binding specificity of the enzyme for (R_P)-UDPαS-Glc and the stereochemical course of the uridylylation process, leading to the inverted configuration in the intermediate.

Authenticity of the Intermediate. The stereochemical results reported here have significance beyond confirming that the double-displacement mechanism involves two inversions at P rather than two retentions. The results also support the authenticity of the uridylyl-enzyme as the true reaction intermediate. The question of authenticity inevitably arises with reference to isolated covalent enzyme–substrate intermediates because of the complexities of enzyme structure and the many possibilities for secondary reactions of isolated intermediates. In this case the problems are compounded by the high chemical reactivities of phosphoramidates and the wealth of nucleophilic functional groups associated with the enzyme, a circumstance that could result in isomerization of the true intermediate. This could involve intramolecular transfer of the uridylyl moiety from the catalytic nucleophile to another or more than one other nucleophilic group during the isolation procedure, resulting in the isolation of a uridylyl-enzyme that is not on the normal catalytic pathway. However, such an isomerization would entail stereochemical consequences at the chiral P. If the uridylylαS-enzyme intermediate isolated in the present work had been epimeric at P, or if it had the same configuration as the substrate, its authenticity as the catalytic intermediate would have been placed in doubt. As it stands, our findings further support the assigned structure of the uridylyl–galactose-1-P uridylyltransferase.

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