THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 269, No. 28, Issue of July 15, pp. 18332–18337, 1994
Printed in U.S.A.

Characterization of GDP-α-D-arabinopyranose, the Precursor of D-Ara$_p$ in *Leishmania major* Lipophosphoglycan*

(Received for publication, February 16, 1994, and in revised form, May 4, 1994)

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Protozoan parasites of the genus *Leishmania* synthesize a complex lipophosphoglycan (LPG), which is the major cell surface macromolecule. The LPG from *Leishmania major* contains β-D-Ara$_p$-terminating side chains that are involved in regulating the attachment of the parasite to the midgut epithelium of its insect vector. An arabinose sugar nucleotide donor was identified in soluble extracts of *L. major* promastigotes. This sugar nucleotide was biosynthetically labeled with D-[2-^{3}H]Glc and with D-[5-^{3}H]Ara. The labeled sugar nucleotide generated [^{3}H]Ara and GDP after mild acid hydrolysis. The absolute configuration of the arabinose was determined after reduction and acylation with a pure enantiomer of Mosher's acid chloride. The pyranose ring configuration was inferred from the ability of GDP-Ara to form borate complexes, and the anomeric configuration was deduced from the results of mild base hydrolysis experiments. Taken together these data suggest that the sugar nucleotide has the structure GDP-α-D-Ara$_p$. This sugar nucleotide has not been previously described from natural sources and may be unique to trypanosomatid protozoan parasites. Ara-1-PO$_4$ and GDP-Ara were the only soluble metabolites labeled with [^{3}H]Ara, and pulse-chase experiment data are consistent with them being precursors of the arabinosyl residues of LPG.

Leishmania major, a protozoan parasite of the Old World, is the causative agent of cutaneous leishmaniasis. The parasite has a digenetic life cycle between a mammalian host, in which it is found as an intracellular amastigote, and the sandfly vector *Phlebotomus papatasi* where it is found in the midgut as the promastigote form (Molyneux and Ashford, 1983). The promastigote forms acquire infectivity through a metacyclogenesis process during which morphological and biochemical changes occur (Sacks, 1989). In particular, there is a significant thickening of the surface glycocalyx that correlates with an increased resistance to complement-mediated lysis, the ability of the promastigotes to detach from the vector midgut epithelium, and promastigote migration to the mouth parts (Puentes *et al.*, 1990; Pimenta *et al.*, 1991). The physical change in the glycocalyx is believed to be due to stage-specific elongation and modification of surface lipophosphoglycan (LPG)[1] (Sacks *et al.*, 1990; Turco and Sacks, 1991; McConville *et al.*, 1992; McConville and Ferguson, 1993). LPG consists of a linear chain of phosphorylated disaccharides, which are attached to the membrane via a glycoinositol phospholipid and are capped with neutral oligosaccharides. Most repeats are substituted with side chains consisting of 1–3 Gal residues that can be capped with a β-D-Ara$_p$ residue (McConville *et al.*, 1990; McConville and Ferguson, 1993). The extent of arabinosylation increases during metacyclogenesis (McConville *et al.*, 1992) and may be important in regulating the detachment of promastigotes from the insect midgut epithelia and the subsequent anterior migration of virulent metacyclic forms (Pimenta *et al.*, 1992).

The biosynthesis of the phosphorylated Galβ1-4Man dissacharide repeat of LPG has been shown in *Leishmania donovani* to involve the transfer of Man-1-phosphate from GDP-Man[2] and Gal from UDP-Gal (Carver and Turco, 1992). UDP-Gal is likely to also be involved in biosynthesis of the Gal side chains of *L. major* LPG. However nothing is known about the activated donor of the uncommon D-Ara$_p$ residue, which has been described so far only in *L. major* and the related trypanosomatids *Crithidia fasciculata* (Gorin *et al.*, 1979; Previato *et al.*, 1982) and *Endotrypanum schaudinni* (Previato *et al.*, 1993). In this report, we characterize GDP-α-D-Ara$_p$, the activated donor for the Ara residue of *L. major* LPG.

EXPERIMENTAL PROCEDURES

Materials—All sugars, nucleotides, and sugar nucleotides were purchased from Sigma. [2-^{3}H]Glc, lot 2949032 (22.8 Ci mmol^{-1}), [6-^{3}H]GlcN (40.4 Ci mmol^{-1}), UDP-[4,5-^{3}H]Gal (39.3 Ci mmol^{-1}), UDP-[6-^{3}H]GlcNAc (6.1 Ci mmol^{-1}), GDP-[3,4-^{3}H]Man (26.4 Ci mmol^{-1}), and NaB^{3}H$_4$ (5–15 Ci mmol^{-1}) were obtained from NEN (Little Chalfont, United Kingdom). Alkaline phosphatase from calf intestine was obtained from Boehringer (Mannheim, Germany) and (*R*)-(−)-α-methoxy-α-(trifluoromethyl)phenylacetyl chloride (Mosher's acid chloride) was from Aldrich. Schneider's *Drosophila* medium minus Glc was from Life Technologies, Inc. Silica Gel 60 HPTLC and cellulose TLC plates were from Merck.

Preparation of [5-^{3}H]Ara—[6-^{3}H]GlcN (100 μCi) was dried and treated with 20 mM ninhydrin in 25 mM citrate-NaOH, pH 4.7, for 40 min at 100 °C (yield about 60%) (Volk, 1960). The reaction mixture was desalted through 200 μl each of AG 50-X12 (H$^+$) over AG 3-X4 (OH$^-$), concentrated, and chromatographed on cellulose TLC as described below. Radioactivity was eluted from the cellulose with water, and the eluate was filtered at 0.2 μm.

Cells—Promastigotes of *L. major* V121 were grown in Schneider's *Drosophila* medium supplemented with 10% fetal calf serum. For biosynthetic labeling experiments, promastigotes in the logarithmic phase of growth were harvested by centrifugation (5 min, 1800 × *g*), washed with labeling medium (Schneider's medium lacking Glc, yeast extract, and fetal calf serum but supplemented with 2.5% bovine serum albumin), resuspended at 2 × 10^8 cells·ml^{-1} in labeling medium plus or minus 2 mM D-ribose, and incubated for 30 min at 27 °C. Label (10 μCi·ml^{-1} [2-^{3}H]Glc or 2.5 μCi·ml^{-1} [5-^{3}H]Ara) was added, and the incubation continued for an additional 1 h at 27 °C. The labeled cells were centrifuged (30 s, 5000 × *g*), and the pellet was immediately extracted on ice as described below. In the pulse-chase experiment, aliquots of 10^8

* This work was supported by the Wellcome Trust. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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[1] The abbreviations used are: LPG, lipophosphoglycan; HPTLC, high performance thin layer chromatography; Ara-ol, arabinitol; Man-ol, mannitol; Gal-ol, galactitol; HPLC, high performance liquid chromatography; SAX, strong anion exchange.

[2] Unless otherwise stated, the sugar is always the D-enantiomer in the pyranose ring configuration and the α anomeric configuration.

cells were removed after 1, 5, 15, 30, and 60 min of continuous labeling and after 5, 15, and 45 min of chase. Chase was performed after 15 min of pulse by transferring part of the cells to a vial containing unlabeled D-Ara (5 mM final).

Extraction of Nucleotides and Soluble Metabolites—Cell pellets were extracted twice with 800 µl of chloroform/methanol/water (2:4:1, v/v) with vortexing and sonication in a Decon FS300 sonicating water bath. The cell pellet volume was assumed to be water in adjusting the proportions of solvents for the first extraction. After centrifugation (3 min at 12,000 × *g*), the pooled supernatants were dried under nitrogen, resuspended in 200 µl of water, and extracted twice with 400 µl of water-saturated 1-butanol. The aqueous phase was dried, redissolved in 1 ml of water, and analyzed by HPLC.

Extraction and Analysis of LPG—Delipidated cell pellets were extracted twice with 400 µl of 9% aqueous 1-butanol with sonication. The pooled supernatants were taken to dryness, redissolved in 5% 1-propanol, 0.1 M ammonium acetate, and loaded onto a 1-ml octyl-Sepharose column equilibrated in the same solvent. The column was washed with 5 volumes of starting solvent and batch eluted with 2 volumes of 40% 1-propanol. The 40% 1-propanol eluate, containing LPG, was freeze-dried. [^{3}H]Ara-labeled LPG was depolymerized (40 mM trifluoroacetic acid, 8 min, 100 °C), and the acid was removed by evaporation. The products were dissolved in 10 mM ammonium bicarbonate and applied to a column (0.5 ml) of QAE-Sephadex A25 equilibrated in the same buffer. The column was washed with 4 ml of 10 mM ammonium bicarbonate, and the phosphorylated repeats were recovered by elution with 2 ml of 1 M ammonium acetate. The phosphorylated repeat fraction was freeze-dried, treated with alkaline phosphatase (4 units in 20 µl of 10 mM ammonium bicarbonate, 16 h, 37 °C), and reduced with $NaBH_4$ (by the addition of 5 µl of 1 N NH_4OH and 10 µl of 1 M $NaBH_4$, 1 h, room temperature). The neutral and reduced LPG repeats were desalted through AG 50-X12 (H^+) (200 µl), dried, redried twice from methanol, desalted through AG 50-X12 (H^+) over AG 3-X4 (OH^-) (100 µl of each) and analyzed by Dionex HPLC as described previously (McConville *et al.*, 1990). Alternatively, the acid-labile β-D-Ara_p residues were directly released from [^{3}H]Ara-labeled LPG by mild acid hydrolysis (40 mM trifluoroacetic acid, 1 h, 100 °C) (McConville *et al.*, 1990), separated from unhydrolyzed material by passage through AG 50-X12 (H^+) over AG 3-X4 (OH^-), and analyzed by Dionex HPLC (see below).

Liquid Chromatography—Nucleotides, sugar nucleotides, and other negatively charged compounds were resolved by HPLC (Merck-Hitachi L-6210) on a Partisil 10-SAX-250A column (4.6 × 250 mm) (HiChrom, Reading, United Kingdom) eluted at 1.5 ml·min^{-1} with a gradient of 5 mM KH_2PO_4 (Buffer A) to 0.5 M KH_2PO_4 (Buffer B). The pH of buffer B was approximately 4.4 and was not adjusted. The eluate was monitored for UV absorbance at 262 nm. The elution gradient was as follows: 0% B for 4 min, then increase to 9% B over 30 min, then to 46% B over 15 min, then to 100% B over 10 min, and finally held for 25 min in 100% B prior to re-equilibration (Pels Rijcken *et al.*, 1990). Fractions (0.6 min, 0.9 ml) were collected, and radioactivity was detected by liquid scintillation counting of aliquots. The system was calibrated with a mixture of 2.5 nmol each of phosphonucleosides and sugar nucleotides injected in the same volume and buffer as the samples. For resolution of sugar nucleotides containing the same nucleotide moiety, the Partisil 10-SAX column was eluted at 1.5 ml·min^{-1} with a gradient of 5 mM $Na_2B_4O_7$ (Buffer A′) to 0.2 M $Na_2B_4O_7$ (Buffer B′). The pH of buffer B′ was about 9.2 and was not adjusted. The elution gradient was as follows: 2 min in 0% B′, then increase to 100% B′ over 40 min, and finally held at 100% B′ for 10 min before re-equilibration. The column was stored in 5 mM KH_2PO_4 immediately after analyses in borate buffers were completed.

Tritiated neutral monosaccharides were resolved by Dionex HPLC using a Carbopack PA1 column (4 × 250 mm) with isocratic elution at 0.6 ml·min^{-1} in 15 mM NaOH (Schneider *et al.*, 1993). Co-injected unlabeled monosaccharide standards (10 nmol each) were detected by pulsed amperometric detection. Radioactivity was detected by liquid scintillation counting.

Chemical and Enzymatic Hydrolyses—Mild acid hydrolyses were performed on the initial aqueous extracts and on relevant peak fractions eluted from the SAX column in phosphate buffer. Samples were adjusted to pH 2.5, by the addition of 1 N H_2SO_4, and heated for 15 min at 100 °C. The pH of the hydrolysate was then adjusted to 7–8 by the addition of a mixture of 5 M NaOH and 1 M acetic acid (2:1, v/v) in order to allow subsequent digestion with alkaline phosphatase (5 units, 6 h at 37 °C).

Base hydrolysis was performed at 60 °C in 110 mM KH_2PO_4/NaOH buffer at pH 11.5, in the presence of 10 mM $NaBH_4$. Aliquots of 115 µl were removed after 0, 10, 30, 60, 120, and 240 min, neutralized with 16 µl of 1 N acetic acid, and diluted to 1 ml with water prior to HPLC analysis. The pH was checked by spotting a small aliquot onto pH paper.

Derivatization of D- and L-Ara—Standards of [1-^{3}H]D-arabinitol and [1-^{3}H]L-arabinitol were prepared by reduction of 5 µmol each of D-Ara and L-Ara, respectively, in 15 µl of 1 N NH_4OH with 5 µl of 30 mM NaB^3H_4 in 0.1 M NaOH for 2 h at room temperature. The reaction mixture was acidified with 15 µl of 1 M acetic acid, desalted through 200 µl of AG 50-X12 (H^+), freeze-dried, redried twice from 50 µl of methanol, desalted through 100 µl each of AG 50-X12 (H^+) over AG 3-X4 (OH^-), dried, and dissolved in 40% 1-propanol. Samples were submitted to descending paper chromatography on Whatman No. 3MM paper for 8 h in 1-butanol/ethanol/water (4:1:0.6, v/v). Arabinitols, the main radioactive products, migrated approximately 5 cm and were eluted from the paper with water.

Biosynthetically labeled GDP-Ara (9000 cpm) purified by SAX HPLC was mixed with 1 nmol each of unlabeled D-arabinitol and L-arabinitol and submitted to mild acid hydrolysis, as described above, desalted through 400 µl each of AG 50-X12 (H^+) over AG 3-X4 (OH^-), dried, redissolved in 20 µl of 1 N NH_4OH, and reduced for 2 h at room temperature with 10 µl of unlabeled 1 M $NaBH_4$. The reaction mixture was then treated as for the standards, except that paper chromatography was omitted. Sample and standards were transferred into 1-ml glass vials with Teflon-lined caps, dried from 20 µl of ethanol, and redissolved in 10 µl of 10% dimethylaminopyridine in pyridine. Samples were derivatized at 65 °C with four successive additions of 20 µl of pyridine and 5 µl of 1 M Mosher's acid chloride in dry chloroform at 1-h intervals. Completeness of the reaction was checked by HPTLC on an aliquot of the standards. The reaction was stopped by the addition of 300 µl of chloroform and 500 µl of saturated $NaHCO_3$. The aqueous phase was discarded, and the organic phase was washed four times with 500 µl of water, evaporated under nitrogen, and then the residue was dissolved in toluene and applied to a 300-µl Silica Gel 60 column equilibrated in toluene. The column was eluted with 4 ml of toluene, and the eluate was taken to dryness. The sample was partitioned between water and toluene to remove residual silica, and the toluene phase was dried and analyzed by Silica Gel 60 HPTLC. The overall yield of the radioactive arabinitol derivative was about 30%.

Thin Layer Chromatography—Monosaccharides were resolved on cellulose TLC with three successive developments in ethyl acetate/pyridine/water (4:2:1, v/v) (Schneider *et al.*, 1993). Unlabeled standards (10 nmol each, except Man, 50 nmol) were detected by silver staining (Chaplin, 1986). Mosher's acid derivatives of arabinitol were analyzed by Silica Gel 60 HPTLC using either ethyl acetate/toluene (2:8, v/v) or toluene to assess completeness of the derivatization. Resolution of the diastereoisomers was obtained by developing the HPTLC 12 times in petroleum ether/toluene (1:1, v/v). Tritiated samples were detected by fluorography after spraying with En^{3}Hance (DuPont NEN).

RESULTS AND DISCUSSION

The structure of the GDP-α-D-Ara sugar nucleotide isolated from *L. major* and the chemical reactions used to support this structure are summarized in Fig. 7.

Isolation of a Novel GDP-containing Sugar Nucleotide—Soluble metabolites of *L. major* V121 were extracted using an acid-free protocol in order to minimize degradation of acid-labile sugar nucleotides. Parasites were extracted with chloroform/methanol/water, and water-soluble metabolites were separated from lipids by phase partitioning between water and 1-butanol. The water-soluble fraction of promastigotes metabolically labeled with commercial [2-^{3}H]Glc was fractionated by anion-exchange chromatography (Fig. 1). Most compounds eluting after 20 min were readily identified as nucleoside di- and triphosphates, NADP, UDP-*N*-acetylhexosamine, UDP-hexose, and GDP-hexose by co-chromatography with authentic standards (Fig. 1) and by their sensitivity or resistance to alkaline phosphatase. The fact that nucleotides incorporated the label suggested that part of the [^{3}H]Glc had been converted to ribose. Indeed, the amount of radioactivity associated with nucleoside di- and triphosphates was reduced by between 30 and 80% when cells were labeled in the presence of 2 mM ribose (data not shown). A relatively minor UV-absorbing compound, eluting shortly after GDP-Man, was consistently labeled to high specific activity (Fig. 1, compound X). This compound was resistant to alkaline phosphatase treatment (data not shown).

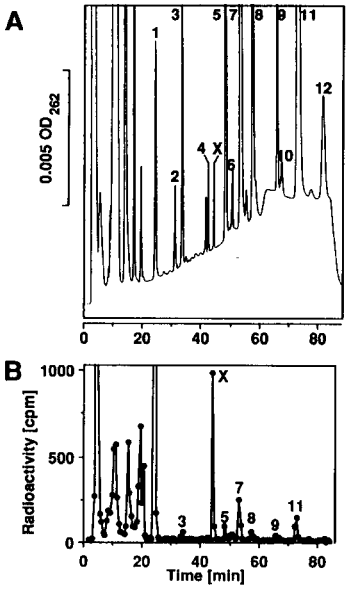

FIG. 1. **Anion-exchange chromatography of *L. major* nucleotide fraction.** Promastigotes were metabolically labeled with commercial [2-^{3}H]Glc in the presence of ribose. Nucleotides in the soluble extract were separated by SAX HPLC. The UV trace and the eluted radioactivity are shown in *panels A* and *B*, respectively. The *peak* marked *X* did not co-chromatograph with any of the sugar nucleotide standards. The *peaks* labeled 1–12 were identified by co-chromatography with authentic standards as: *peak 1*, GMP; *peak 2*, UDP-HexNAc; *peak 3*, UDP-Hex; *peak 4*, GDP-Hex; *peak 5*, UDP and NADP; *peak 6*, CDP; *peak 7*, ADP; *peak 8*, GDP; *peak 9*, UTP; *peak 10*, CTP; *peak 11*, ATP; *peak 12*, GTP.

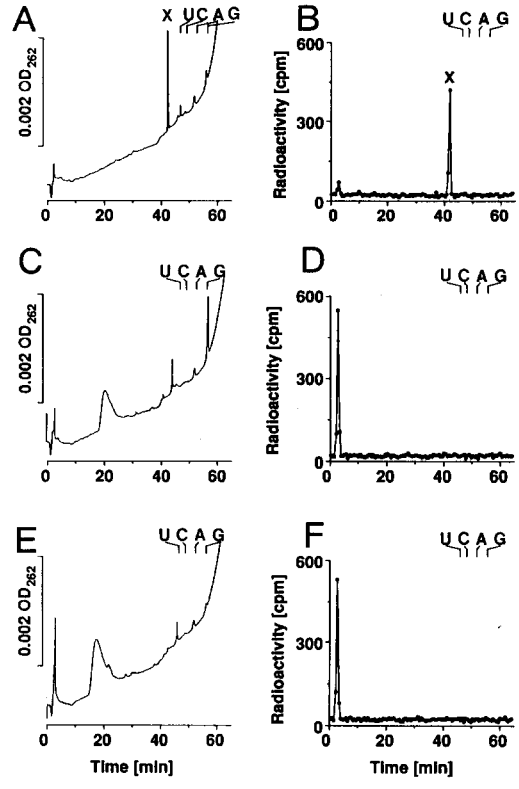

FIG. 2. **Identification of the nucleotide moiety of compound X as GDP.** Biosynthetically labeled compound X (Fig. 1) was rechromatographed by SAX HPLC before (*panels A* and *B*) and after (*panels C* and *D*) mild acid hydrolysis. The mild acid-hydrolyzed material was also analyzed by SAX HPLC after digestion with alkaline phosphatase (*panels E* and *F*). The elution positions of UDP, CDP, ADP, and GDP are indicated by *U*, *A*, *C*, and *G*, respectively. UV absorbance at 262 nm is shown in *panels A*, *C*, and *E*. Radioactivity was monitored by liquid scintillation counting (*panels B*, *D*, and *F*).

Compound X was completely hydrolyzed by mild acid treatment to yield a neutral radioactive product and a negatively charged UV-absorbing product (Fig. 2). The latter compound co-chromatographed with authentic GDP and was sensitive to alkaline phosphatase (Fig. 2). From these results, we deduce that compound X is a sugar nucleotide consisting of a neutral labeled sugar in glycosidic linkage to the β-phosphate of guanosine diphosphate. Such a structure is consistent with the elution of compound X close to GDP-Man.

The Novel Sugar Nucleotide Is GDP-Ara—Radioactivity released from compound X by mild acid hydrolysis co-chromatographed with authentic Ara on both cellulose TLC and Dionex HPLC and was clearly resolved from a range of other neutral monosaccharides (Fig. 3). These results define the sugar nucleotide as GDP-Ara.

The Ara Residue Is in the Pyranose Ring Form—The ring structure (pyranose or furanose) of the Ara residue was deduced by anion-exchange chromatography in the presence of borate ions. The presence of *cis*-hydroxyl groups in a monosaccharide promotes the formation of boronate complexes, which are negatively charged and therefore retarded by anion-exchange chromatography. Thus, while GDP-Man and GDP-Glc co-chromatograph by conventional SAX HPLC eluted with phosphate buffer, GDP-Man (*cis*-hydroxyl groups at C-2 and C-3) is retarded with respect to GDP-Glc (no *cis*-hydroxyl groups) by SAX HPLC eluted with borate buffer. Chromatography of GDP-[^{3}H]Ara by SAX HPLC in the presence of borate ions showed that it eluted much closer to GDP-Man than to GDP-Glc, strongly suggesting that it contains *cis*-hydroxyl groups[3] (Fig. 4). This suggests that the Ara residue is in the pyranose configuration (Ara_p contains *cis*-hydroxyl groups at C-3 and C-4 whereas Ara_f contains no *cis*-hydroxyl groups). The structure of the sugar nucleotide could therefore be refined to GDP-Ara_p.

The Ara_p Residue Is the α-Anomer—The anomeric configuration of the Ara_p residue was inferred from the products of mild base hydrolysis in the presence of $NaBH_4$. There are three distinct mechanisms of base-catalyzed sugar nucleotide hydrolysis, all of which are dependent on the presence of an hydroxyl group at C-2 adjacent to the phosphodiester group. When these groups are *cis*, the initial reaction products are nucleoside monophosphate and a sugar 1,2-cyclic phosphate. The latter product slowly undergoes ring opening to form a mixture of aldose 1-phosphate and alditol 2-phosphate. UDP-Gal and GDP-Glc are hydrolyzed by this mechanism, whereas UDP-GlcNAc (which has no hydroxyl group at C-2) is base stable (Table I and Fig. 5*A*). When the phosphodiester and the C-2 hydroxyl groups are *trans*, there will be an equilibrium mixture of *trans* diaxial and *trans* diequatorial forms, due to the interconversion of the $^4C_1$ and the $^1C_4$ ring conformations of the sugar. Each of these forms will undergo a different hydrolysis reaction. The *trans* diaxial form will hydrolyze via a 1,2-epoxide intermediate (Cawley and Letters, 1971) to yield a nucleoside diphosphate and an alditol. The *trans* diequatorial form will hydrolyze via a 1,2-cyclic phosphate intermediate to yield a nucleoside monophosphate and a mixture of aldose

[3] Anion-exchange chromatography in borate buffer proved to be useful to purify compound X extracted from promastigotes grown in complete medium in the presence of fetal calf serum. Indeed, when promastigotes were grown under these conditions, one or several compounds coeluted with compound X during anion exchange in phosphate buffer. The nature of this contamination has not been determined, but it eluted before compound X during a second step of anion-exchange chromatography in borate buffer and could therefore be removed (data not shown).

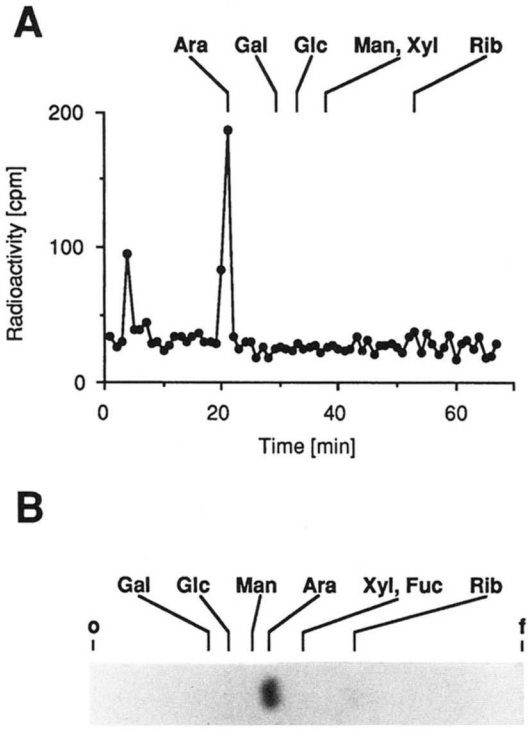

FIG. 3. **Identification of the monosaccharide moiety of compound X as Ara.** Biosynthetically labeled compound X (Fig. 1) was submitted to mild acid hydrolysis, and the ^{3}H-labeled neutral monosaccharide product was analyzed by Dionex HPLC (*panel A*) and cellulose TLC (*panel B*). Radioactivity was detected by liquid scintillation counting and fluorography, respectively. The elution or migration positions of various neutral monosaccharide standards are indicated in both cases *Xyl*, xylose; *Fuc*, fucose.

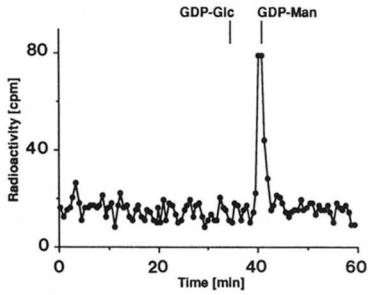

FIG. 4. **Determination of the conformation of the Ara residue in GDP-Ara by SAX HPLC in borate buffer.** Biosynthetically labeled compound X (Fig. 1) was rechromatographed on SAX HPLC using a borate buffer system. Radioactivity was detected by liquid scintillation counting. The elution positions of co-injected unlabeled GDP-Glc and GDP-Man internal standards, detected by UV absorbance, are indicated.

1-phosphate and alditol 2-phosphate.[4] The ratio of the final products is dependent on the relative energies of the transition states of the two reactions, which in turn is dependent on the nature of the sugar. GDP-Man is hydrolyzed through these mechanisms and thus yields three carbohydrate products: mannitol, mannitol 2-phosphate, and Man-1-phosphate (Table I and Fig. 5*B*).

The results of the base hydrolysis of purified GDP-Ara$_p$ are shown in Fig. 5*C* and Table I. GDP was the main UV-absorbing product, and three ^{3}H-labeled carbohydrate products were identified: arabinitol (75%), arabinitol 2-phosphate (5%), and Ara-1-phosphate (20%). Generation of these products can only be explained if the Ara$_p$ residue is in the α anomeric configu-

[4] The ratio of aldose 1-phosphate to alditol 2-phosphate is 4:1 upon the opening of the 1,2-cyclic phosphate when this is *trans* diequatorial. This ratio is reversed when the 1,2-cyclic phosphate intermediate is *cis* (see Table I).

TABLE I
Products of mild base hydrolysis in the presence of NaBH$_4$, of GDP-Ara, and of other nucleotide sugars

Nucleotide sugar	$t_{1/2}$	Main UV-absorbing product	Carbohydrate products[a]
	min		
UDP-GlcNAc	>10^5		
GDP-Glc	8	GMP	ND[b]
UDP-Gal	8	UMP	Gal-1,2-cyclic-PO$_4$[c]
			Gal-1-PO$_4$ (20%)
			Gal-ol-2-PO$_4$ (80%)
GDP-Man	90	GDP	Man-ol (90%)
			Man-1-PO$_4$ (8%)
			Man-ol-2-PO$_4$ (2%)
GDP-Ara	12	GDP	Ara-ol (75%)
			Ara-1-PO$_4$ (20%)
			Ara-ol-2-PO$_4$ (5%)

[a] Characterized by their retention time on SAX HPLC, susceptibility or resistance to alkaline phosphatase and mild acid treatment, and by comigration of the released neutral radioactivity with aldose and alditol standards on Dionex HPLC.

[b] Not determined.

[c] Intermediate reaction product representing up to 70% of the total radioactivity in the early time points (see Fig. 5).

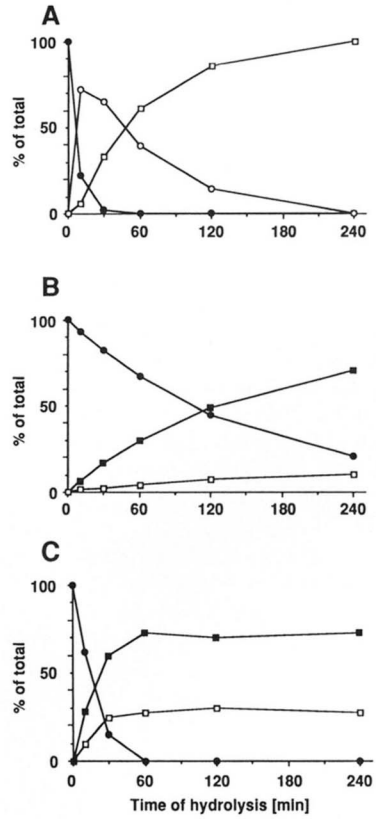

FIG. 5. **Determination of the anomeric configuration of the Ara residue in GDP-Ara.** UDP-[^{3}H]Gal and GDP-[^{3}H]Man standards, and GDP-[^{3}H]Ara were subjected to mild base hydrolysis in the presence of NaBH$_4$ (*panels A*, *B*, and *C*, respectively). At various times, aliquots of the hydrolysates were separated by SAX HPLC, and the ratios of sugar nucleotide (*filled circles*), sugar 1,2-cyclic phosphate (*open circles*), sugar monophosphate (*open squares*), and neutral sugar (*filled squares*) were determined. The identities of the sugar cyclic phosphate and sugar monophosphates are described in Table I. The GDP-[^{3}H]Ara sample contained unlabeled internal standards of GDP-Man and UDP-Gal, which released UV-absorbing GDP and UMP upon hydrolysis, respectively (not shown). In this particular sample, the $t_{1/2}$ of GDP-Man was 90 min, and the $t_{1/2}$ of UDP-Gal was 8 min.

ration. The structure of the sugar nucleotide could therefore be further refined to GDP-α-Ara_p.

It is worth noting that GDP-Ara_p undergoes more rapid hydrolysis than GDP-Man (Table I). Presumably, the lack of an exocyclic C-6 hydroxymethyl group in Ara_p confers greater ring flexibility and thus may potentiate the formation of the 1,2-epoxide intermediate.

The Ara Residue Is in the D Configuration—The absolute configuration of the Ara residue (D or L) was determined after reduction of mild acid-released [^{3}H]Ara to [^{3}H]arabinitol and derivatization with a pure enantiomer of Mosher's acid chloride. The Mosher's acid derivatives of D- and L-arabinitol are diastereoisomers that can be resolved by Silica Gel 60 HPTLC. The [^{3}H]arabinitol derivative co-migrated with the authentic D-arabinitol derivative and not with the L-arabinitol derivative (Fig. 6). The structure of the sugar nucleotide could therefore be refined to GDP-α-D-Ara_p (Fig. 7).

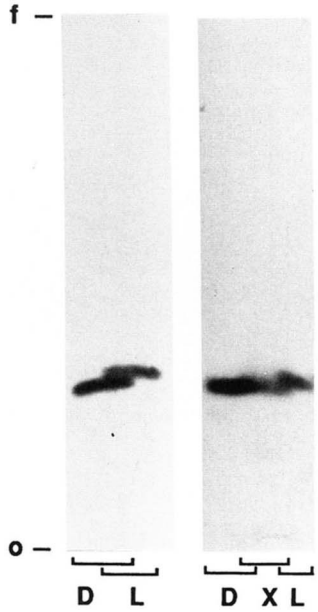

FIG. 6. **Determination of the absolute configuration of the Ara residue in GDP-Ara.** [^{3}H]-labeled Ara was released from GDP-Ara by mild acid hydrolysis, reduced, acylated with optically active Mosher's acid chloride, purified, and analyzed by HPTLC. This sample (*lane X*) was applied to the HPTLC plate together with identically processed standards of [^{3}H]L-arabinitol and [^{3}H]D-arabinitol (*lanes D* and *L*, respectively). Radioactivity was detected by fluorography.

GDP-Ara, Ara-1-PO_4, and LPG Are Biosynthetically Labeled with [5-^{3}H]D-Ara—When cells were labeled with [2-^{3}H]Glc (see Fig. 1) the GDP-Ara was found to have a surprisingly high specific activity as compared with the other sugar nucleotides. However, we found by cellulose TLC and Dionex HPLC that approximately 3% of the commercial Glc preparation was in fact [^{3}H]Ara (most likely [1-^{3}H]D-Ara), suggesting that a large proportion of the label in GDP-Ara came from this trace contaminant. Indeed, GDP-Ara could be easily labeled with synthetic [5-^{3}H]D-Ara (Fig. 8).

Only one other soluble metabolite was labeled with [5-^{3}H]Ara. This compound eluted very close to GMP but did not display any measurable absorbance at 262 nm (data not shown). This latter compound was sensitive to alkaline phosphatase as well as to mild acid hydrolysis, yielding neutral Ara in both cases. In addition, the compound yielded Ara when it was first reduced with $NaBH_4$ and then submitted to mild acid hydrolysis but yielded arabinitol when submitted to the same reactions in the reverse order. These data indicate that this compound is Ara-1-PO_4. No apolar lipid was labeled with [5-^{3}H]Ara, making it very unlikely that dolichol phosphate-type activated sugar donors are involved in the transfer of arabinosyl residues to LPG (data not shown).

In addition to Ara-1-PO_4 and GDP-Ara, LPG was biosynthetically labeled with [5-^{3}H]Ara under the same conditions. When LPG was subjected to mild acid hydrolysis, under conditions known to release the acid labile β-D-Ara_p residues of LPG (McConville *et al.*, 1990), greater than 80% of the radioactivity became neutral and comigrated with Ara on Dionex HPLC (data not shown). Dephosphorylated and reduced repeats of the LPG were also prepared. These radiolabeled oligosaccharitols were resolved by Dionex HPLC into two components (in a ratio of 4:1) that coeluted with authentic standards of Araβ1-2Galβ1-3Galβ1-4Man-ol and Araβ1-2Galβ1-3Galβ1-3Galβ1-4Man-ol, respectively. No radioactivity was detected at the elution positions of non-arabinosylated repeats (McConville *et al.*, 1990). These data demonstrate that the LPG was labeled only in its arabinosyl residues.

Ara-1-PO_4 and GDP-Ara Are Precursors of Arabinosyl Residues in LPG—Promastigotes were labeled with [5-^{3}H]Ara for 15 min and then either labeled for a further 45 min or chased

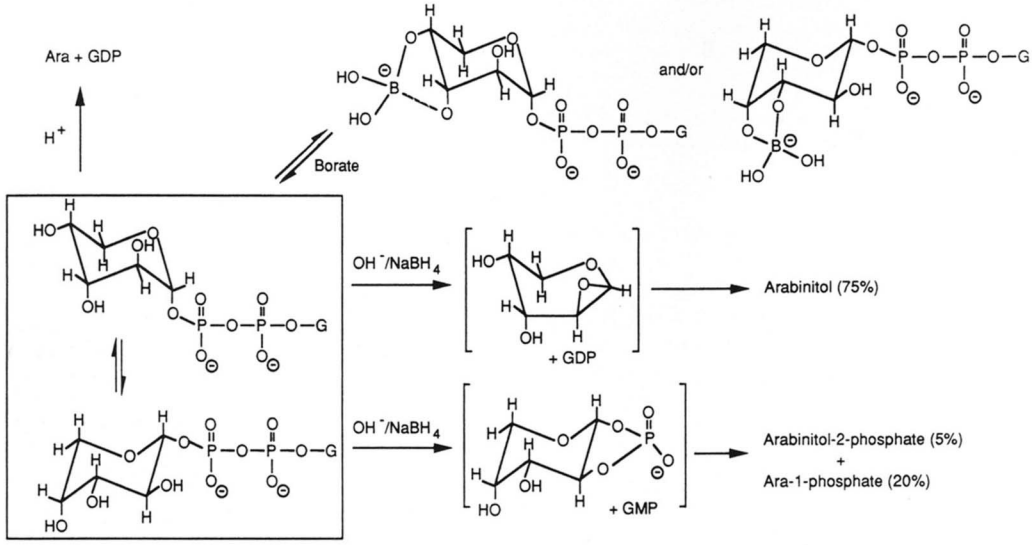

FIG. 7. **Proposed structure of the GDP-α-D-Ara_p sugar nucleotide.** GDP-α-D-Ara_p is shown in its $^4C_1$ (*top*) and $^1C_4$ (*bottom*) conformations. The products of mild acid hydrolysis (see Figs. 2, 3, and 6), borate complexation (see Fig. 4), and mild base hydrolysis (see Fig. 5) are shown. The structures shown in *square brackets* are putative short-lived intermediates that have not been directly characterized.

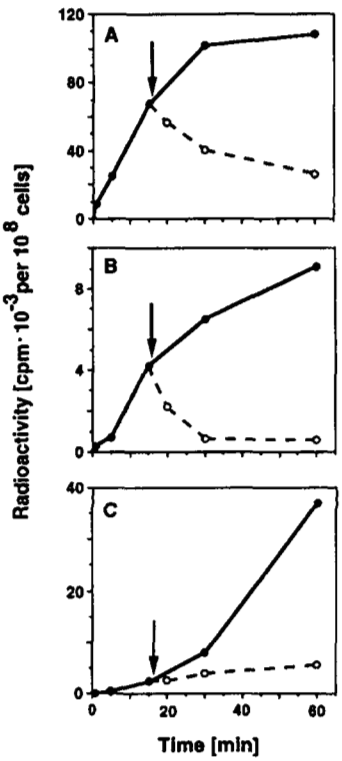

FIG. 8. **Pulse-chase labeling and precursor-product relationships of Ara-1-PO_4, GDP-Ara, and LPG.** Promastigotes were metabolically labeled with [5-^{3}H]Ara, and aliquots were removed from the labeling reaction for analysis at the indicated times (*closed circles*). After 15 min of pulse, part of the cells were chased (*open circles*) with 5 mM D-Ara, as indicated by the *arrows*. Soluble metabolites were chromatographed and detected as shown in Fig. 1. LPG was isolated by octyl-Sepharose chromatography. *Panel A*, Ara-1-PO_4; *panel B*, GDP-Ara; *panel C*, LPG.

with unlabeled D-Ara (Fig. 8). In the continuous labeling experiment, Ara-1-PO_4 was the first labeled product to appear, reaching steady-state labeling after about 30 min. GDP-Ara appeared slightly later and only approached steady-state labeling after about 60 min. The kinetics of LPG labeling were significantly slower and showed no signs of approaching a steady state over the time course of the experiment.

Both Ara-1-PO_4 and GDP-Ara could be chased upon the addition of unlabeled D-Ara, indicating that they are rapidly turned over, as expected for biosynthetic intermediates. The rapid decline in labeled GDP-Ara levels correlated well with the dramatic reduction in the rate of LPG labeling, further suggesting that GDP-Ara is the immediate donor of Ara in the arabinosylation of LPG.

Summary—Ara_p-containing sugar nucleotides are known to occur in plants and brown algae (Feingold and Barber, 1990). These sugar nucleotides are UDP-β-L-Ara_p (described in plants and brown algae) and ADP-β-L-Ara_p and GDP-β-L-Ara_p (described in certain species of brown algae). However, these molecules are based on L-Ara_p rather than D-Ara_p and, to our knowledge, the latter sugar is unique to a few trypanosomatid protozoan parasites. The small quantities of the novel sugar nucleotide present in the *L. major* promastigote cell extracts (approximately 0.8 nmol/10^{10} cells, based on UV absorbance) prevented direct chemical analysis by techniques such as NMR. However, the data presented here strongly suggest that the structure of this sugar nucleotide is GDP-α-D-Ara_p (Fig. 7). We believe that this is the first description of this sugar nucleotide or indeed of any activated D-Ara donor from natural sources. This sugar nucleotide is used by the parasite to add the terminal β-D-Ara_p residues found in *L. major* LPG. The identification of this donor opens the way for its chemical synthesis and for subsequent purification and characterization of the parasite β-arabinosyltransferase(s).

Acknowledgments—We thank Dr. Andrey Nikolaev, Dr. Robert Field, and Dr. Thomas Ilg for helpful suggestions and Prof. John Brimacombe for critical reading of the manuscript.

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