The biosynthesis of GDP-D-arabinopyranose in *Crithidia fasciculata*: characterization of a D-arabino-1-kinase activity and its use in the synthesis of GDP-[5-³H]D-arabinopyranose

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GDP-D-arabinopyranose (GDP-D-Ara) is the precursor of the uncommon D-arabinopyranose residues present in the glycoconjugates of a few trypanosomatid parasites. Biosynthetic labelling experiments with *Crithidia fasciculata* showed that GDP-D-Ara could be labelled with [³H]D-Ara, [2-³H]D-Glc and [6-³H]D-Glc, but not with [1-³H]D-Glc, suggesting that D-Ara can be either taken up directly by the parasite or derived from D-Glc through a pathway involving the loss of carbon C-1. *In vivo* pulse-chase experiments indicated that D-Ara was sequentially incorporated into D-Ara-1-PO₄ and GDP-D-Ara prior to transfer to the acceptor glycoconjugate, lipoarabinogalactan. An MgATP-dependent D-arabino-1-kinase activity present in soluble

extracts of *C. fasciculata* was purified away from phosphatase activities by size-exclusion chromatography. The D-arabino-1-kinase had an apparent molecular mass of 600 kDa, a neutral optimum pH, and displayed substrate inhibition at D-Ara concentrations above 100 µM. It had a K_m^{ATP} of 1.7 mM and a K_m^{Ara} of 24 µM. Competition studies indicated that the orientation of every single hydroxyl residue was important for D-Ara recognition by the enzyme, but that methyl or hydroxymethyl groups could be tolerated as equatorial substituents on C-5 of D-Ara. The partially purified D-arabino-1-kinase activity was used in the chemico-enzymic synthesis of GDP-[5-³H]D-Ara from [6-³H]D-GlcN.

INTRODUCTION

The pentose arabinose (Ara) is widely used in plants and in some bacteria, principally as a constituent of cell wall glycoconjugates. Unlike most other monosaccharides, arabinose occurs naturally in all combinations of ring and absolute configurations, namely L-Ara_p, L-Ara_f, D-Ara_p and D-Ara_f. The rare D-Ara_p has only been described in a few protozoan trypanosomatid parasites. It occurs in a glycoinositolphospholipid of *Endotrypanum schaudinni* [1], in a D-arabino-D-galactan of *Crithidia fasciculata* [2,3] and in the complex lipophosphoglycan (LPG) of *Leishmania major* (reviewed in [4]). *C. fasciculata* is a monogenetic parasite that normally lives in the gut of various fly hosts, and that can be easily grown axenically. *L. major*, which is the causative agent of cutaneous leishmaniasis, has a digenetic life cycle between a phlebotomine sandfly vector and a mammalian host in which it resides within the acidified phagolysosomes of infected macrophages. LPG is mainly expressed at the surface of the insect stage of the parasite (the promastigote form) and it is an important component of the glycocalyx which covers this flagellated cell. During metacyclogenesis, which is the process through which promastigotes acquire infectivity [5], two significant structural changes occur in the LPG molecule: (a) an approximate doubling of the number of phosphorylated oligosaccharide repeats that constitute the backbone of the molecule, and (b) a dramatic increase in the extent of terminal arabinosylation of the β-Gal-terminating side-chains [6]. These structural changes correlate with a pronounced thickening of the glycocalyx [7] and with the

detachment of the metacyclic promastigotes from the midgut epithelium of the vector, followed by forward migration to the mouth parts. It has been suggested that the capping of LPG side-chains with D-Ara_p is directly involved in parasite detachment, probably by preventing the underlying D-Gal_p residues from interacting with gut lectins [8].

The activated donor of D-Ara_p has been identified in *L. major* as the sugar nucleotide GDP-α-D-Ara_p [9]. In the present study, the pathway of GDP-D-Ara biosynthesis has been investigated in the related trypanosomatid *C. fasciculata*. One of the biosynthetic enzymes, arabinose-1-kinase, has been identified, characterized and used in the chemico-enzymic synthesis of radiolabelled GDP-D-Ara.

MATERIALS AND METHODS

Materials

[6-³H]D-Glc (50 Ci·mmol⁻¹), [1-³H]D-Glc (10 Ci·mmol⁻¹), [2-³H]D-Glc (22.8 Ci·mmol⁻¹) and [6-³H]D-GlcN hydrochloride (36–40 Ci·mmol⁻¹) were purchased from DuPont-NEN (Little Chalfont, Bucks., U.K.). Alkaline phosphatase from calf intestine was obtained from Boehringer (Mannheim, Germany). GMP-morpholidate (*N,N'*-dicyclohexyl-4-morpholine carboxamidinium salt) and proteins for gel-permeation chromatography calibration were purchased from Sigma. Schneider's *Drosophila* medium (minus Glc) was from Life Technologies. Silica gel 60 HPTLC

Ara, arabinose; GDP-D-Ara, GDP-D-arabinopyranose; GPI, glycosylphosphatidylinositol; HPAEC, high-pressure anion-exchange chromatography; LAG, lipoarabinogalactan; LPG, lipophosphoglycan; D-Rib, D-ribose; SAX, strong-anion-exchange.

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and cellulose TLC plates were from Merck (Darmstadt, Germany). Pyridine was distilled over calcium hydride just before use.

Purification of labels

Commercial batches of [^3H]D-Glc and [^3H]D-GlcN contain a proportion of [^3H]D-Ara contaminant which was removed prior to biosynthetic labelling. Radioactive GlcN and Glc were applied to cellulose TLC plates (about $100\ \mu\text{Ci}\cdot\text{cm}^{-1}$) and submitted to two developments in ethyl acetate/pyridine/water (4:2:1, by vol.). Radioactivity was eluted from the cellulose with three washes of water and filtered through a $0.2\ \mu\text{m}$ -pore-size membrane. Alternatively, [^3H]D-Glc was separated from contaminating [^3H]D-Ara by Dionex high-pressure anion-exchange chromatography (HPAEC), as described below. The radiochemical purity of [^3H]D-Glc was checked by Dionex HPAEC.

Cell culture

C. fasciculata strain HS6 was maintained at 27°C in Schneider's *Drosophila* medium supplemented with 5% fetal-calf serum, and diluted twice weekly. For larger cultures, cells were grown at 27°C in modified Schaefer's medium as described previously for *L. major* [10,11], except that BSA and fetal-calf serum were omitted.

Biosynthetic labelling and extraction of soluble metabolites

C. fasciculata cultures were diluted (1 in 10) 16 h prior to biosynthetic labelling. Cells were harvested by centrifugation (4°C , 5 min, 1800 g) and washed in ice-cold labelling medium (Schneider's *Drosophila* medium lacking Glc, yeast extract and fetal-calf serum, but supplemented with 2.5% BSA). Labellings were initiated by the addition of the label {typically $20\ \mu\text{Ci}$ of [^3H]D-Glc per ml or $0.5\ \mu\text{Ci}$ of [^3H]D-Ara per ml, plus or minus 2 mM of unlabelled D-ribose (D-Rib) and/or D-Ara or L-Ara, in $500\ \mu\text{l}$ of labelling medium at 27°C } directly to ice-cold cell pellets, and the mixtures were transferred to 20 ml glass vials. Cells were incubated for 20 min, with occasional agitation, in a water bath held at 27°C and then harvested in 1.5 ml polypropylene tubes (30 s, 5000 g). Pellets were extracted twice on ice with $500\ \mu\text{l}$ of chloroform/methanol/water (1:2:0.5 and 1:2:0.8, by vol., for the first and second extractions respectively) and the pooled supernatants taken to dryness. The mixture was partitioned twice in a two-phase system between $400\ \mu\text{l}$ of water-saturated 1-butanol and $200\ \mu\text{l}$ of 1-butanol-saturated water. The aqueous phase, containing the soluble metabolites, was dried and resuspended in water prior to analysis by strong-anion-exchange (SAX) HPLC. In some cases, the extracted cell pellets were dried briefly under N_2 and re-extracted twice with 9% 1-butanol in water, with sonication in a sonicating water bath. The pooled supernatants were taken to dryness, redissolved in 0.1 M ammonium acetate containing 5% 1-propanol and applied to a $300\ \mu\text{l}$ column of octyl-Sepharose in the same buffer. The column was washed with 15 vol. of the same buffer and eluted with $500\ \mu\text{l}$ of 40% 1-propanol followed by $500\ \mu\text{l}$ of 50% 1-propanol. Lipoarabinogalactan (LAG) was recovered in the pooled 1-propanol eluates.

For the pulse-chase experiment, cells (9×10^8 in 1.4 ml) were preincubated for 2 min at 27°C . At time zero, $9\ \mu\text{Ci}$ of [^3H]D-Ara in $100\ \mu\text{l}$ of labelling medium was added. After 60 s, $500\ \mu\text{l}$ of the culture was transferred into another vial and made 5 mM with respect to D-Ara. Aliquots ($160\ \mu\text{l}$) were removed from the continuous labelling reaction (at 5, 15, 50, 80 and 110 s) and from the chase reaction (at times 65, 75 and 115 s) and added

directly to $600\ \mu\text{l}$ of ice-cold chloroform/methanol (1:2, v/v). The cell pellet was extracted once more with chloroform/methanol/water (1:2:0.8, by vol.) and the pooled supernatants taken to dryness and processed as described above.

Liquid chromatography

Soluble metabolite extracts were loaded on to a Partisil 10-SAX-250A HPLC column ($4.6\ \text{mm} \times 250\ \text{mm}$, HiChrom, Reading, U.K.) equilibrated in 5 mM KH_2PO_4 , pH 4.4 (buffer A), and eluted at $1.5\ \text{ml}\cdot\text{min}^{-1}$ with a multi-step gradient to 500 mM KH_2PO_4 (buffer B). The following elution gradient was used: 0% buffer B for 4 min, 0–10% buffer B over 33 min, 10–46% buffer B over 15 min, 46–100% buffer B over 10 min followed by 20 min at 100% buffer B. UV absorbance was monitored on-line at 262 nm, and fractions (1.5 ml) were collected. Radioactivity was detected by liquid scintillation counting of a tenth of the fractions.

Neutral monosaccharides were resolved by Dionex HPAEC using a CarboPack PA-1 column ($4\ \text{mm} \times 250\ \text{mm}$) eluted isocratically at $0.6\ \text{ml}\cdot\text{min}^{-1}$ with 15 mM NaOH. The elution positions of internal monosaccharide standards (50 pmol each of D-Ara, D-Glc, D-Gal, D-Man and D-arabinitol) were monitored by pulsed amperometric detection and radioactivity was detected by liquid scintillation counting of the fractions.

Chemical and enzymic treatments

For mild acid hydrolysis, fractions from the SAX column were adjusted to pH 2–2.5 with 0.5 M H_2SO_4 and incubated for 15 min at 100°C . For alkaline phosphatase treatment, samples were adjusted to pH 8 with a mixture of 5 M NaOH and 1 M acetic acid (2:1, v/v) and incubated with 25 units $\cdot\text{ml}^{-1}$ of enzyme for 16 h at 37°C . For reduction, samples were adjusted to pH 9 and reduced with 10 mM NaBH_4 for 30 min at room temperature. Excess reductant was destroyed with H_2SO_4 . Reaction mixtures were diluted 4-fold with water prior to analysis by SAX-HPLC. Neutral monosaccharides were desalted by passage through AG50-X12 (H^+) over AG3-X4 (OH^-) ($400\ \mu\text{l}$ each) and analysed by Dionex HPAEC as described above.

Nitrous acid deamination was performed in 0.2 M sodium acetate (pH 4.0)/0.33 M NaNO_2 (deamination buffer) for 3 h at 60°C , with two additions of fresh deamination buffer at 1 h intervals. In a control incubation, NaNO_2 was replaced by NaCl.

Mild acid hydrolysis of LAG was performed in 40 mM trifluoroacetic acid/1-propanol (4:1, v/v) for 1 h at 100°C . Monosaccharides were released by strong acid hydrolysis in 2 M trifluoroacetic acid (2 h, 100°C).

Extraction of a D-arabino-1-kinase activity

C. fasciculata (10^9 cells; 0.1 ml of packed cells) were submitted to two cycles of freezing and thawing in $600\ \mu\text{l}$ of 10 mM Tris/HCl, pH 7.5, with or without the following protease inhibitors: $10\ \mu\text{g}\cdot\text{ml}^{-1}$ each leupeptin, soya trypsin inhibitor and pepstatin, $250\ \mu\text{M}$ tosyl-lysylchloromethane ('TLCK'), $250\ \mu\text{M}$ PMSF, 1 mM 1-10 phenanthroline and 1 mM iodoacetamide. (No difference was observed in the activity or apparent molecular mass of the D-arabino-1-kinase activity whether or not protease inhibitors were included in the extraction mixture. Therefore these were not included in subsequent extractions of D-arabino-1-kinase.) The extract was centrifuged twice ($10000\ \text{g}$, 1 min, 4°C) and the supernatant was filtered through a $0.4\ \mu\text{m}$ membrane and $500\ \mu\text{l}$ was loaded on to a Superdex-200 HR 10/30 column ($10\ \text{mm} \times 300\ \text{mm}$, Pharmacia), equilibrated and eluted at $0.4\ \text{ml}\cdot\text{min}^{-1}$ in 50 mM $\text{NaH}_2\text{PO}_4/\text{NaOH}$ (pH 6.7) containing

10 mM MgSO_4 and 100 mM KCl. The column was calibrated with Blue Dextran (> 2000 kDa), thyroglobulin (669 kDa), apoferritin (443 kDa), alcohol dehydrogenase (200 kDa), β -amylase (150 kDa), BSA (66 kDa), ovalbumin (43 kDa), carbonic anhydrase (29 kDa) and cytochrome *c* (12.4 kDa). Phosphatase activities were detected with 2 mM *p*-nitrophenol phosphate in 10 mM Tris/HCl, pH 7.5, 140 mM NaCl. D-Arabinose-1-kinase activity was detected as described below. Fractions containing the kinase activity were pooled and kept frozen or freeze-dried at -70°C . No major loss of activity was noticed after seven cycles of freezing and thawing, or after freeze-drying, but the activity was lost in a few days when stored at 4°C .

Enzymic assay for D-arabino-1-kinase

D-Arabinose-1-kinase activity was assayed as follows: 2 vol. of crude extract or column fractions were added to 1 vol. of a mixture containing 100 mM NaF, 2 mM ATP, 10 mM MgSO_4 , 50 mM $\text{NaH}_2\text{PO}_4/\text{NaOH}$ (pH 6.7) and $30\ \mu\text{Ci}\cdot\text{ml}^{-1}$ of $[5\text{-}^3\text{H}]\text{D-Ara}$. Monosaccharides at various concentrations were included in some experiments. The mixture was incubated for 15 to 30 min at 30°C and reaction was terminated on ice by the addition of 1 vol. of 1-propanol and 1 vol. of 100 mM Na-EDTA, pH 8. An aliquot of the reaction mixture (5 μl) was applied to silica HPTLC (5 cm) and developed once in chloroform/methanol/water (10:10:3, by vol.) (developing time of about 12 min). Radioactivity was detected by fluorography (16 h, -70°C) after spraying with En³Hance (DuPont-NEN). For quantification, areas containing D-Ara and D-Ara-1- PO_4 were localized precisely using the fluorogram, scraped, mixed with 300 μl of water and counted by liquid scintillation.

Determination of the pH optimum and K_m

A buffer containing 10 mM MgSO_4 and 100 mM each of acetic acid, NaH_2PO_4 and Tris-base was brought to pH 4 with conc. HCl. The buffer was then adjusted to pH values in the range of 4–9.5 with 5 M NaOH. These buffers (1 vol.) were mixed with 2 vol. of $20\ \mu\text{M}$ D-Ara, $7.5\ \mu\text{Ci}\cdot\text{ml}^{-1}$ of $[5\text{-}^3\text{H}]\text{D-Ara}$ and the mixture was taken to dryness. D-Arabinose-1-kinase (1 vol. of the pool of the Superdex-200 active fractions) was added to the dried buffers and warmed for 1 min at 30°C . Reaction was initiated by the addition of 1 vol. of 1.2 mM ATP (at 30°C) and aliquots (0.5 vol.) of the reaction were removed after 30, 60 and 120 s and added to tubes containing 0.12 vol. of 0.1 M Na-EDTA (pH 8) and 0.12 vol. of 1-propanol. The pH in the reaction mixture was checked on pH paper (accuracy of 0.2 to 0.3 pH unit). Samples (5 μl) of the reaction mixtures were analysed by HPTLC as described above. Reaction rates were linear over the first 2 min.

A similar protocol was used for the determination of kinetic constants. In this case, the buffer was 50 mM $\text{NaH}_2\text{PO}_4/\text{NaOH}$, pH 6.7, containing 10 mM MgSO_4 and 100 mM KCl, with various concentrations of ATP (0.3–2.5 mM) and D-Ara (5–400 μM). The reactions also contained $[5\text{-}^3\text{H}]\text{D-Ara}$ at a concentration of 0.27 μM (assuming that the specific radioactivity of $[5\text{-}^3\text{H}]\text{D-Ara}$ is equal to that of the $[6\text{-}^3\text{H}]\text{D-GlcN}$ from which it was derived, i.e. $36.7\ \text{Ci}\cdot\text{mmol}^{-1}$). Under these conditions, most of the ATP is complexed with Mg^{2+} [12]. Experimental data obtained with D-Ara concentrations up to 100 μM were plotted in double-reciprocal Lineweaver–Burk plots. Values obtained by linear regression for the slopes and intercepts were replotted against the inverse of the concentrations and fitted by linear regression. Values for V_{max} , K_m^{Ara} and K_m^{ATP} were extracted from the slopes and intercepts of these replots [13,14].

Chemico-enzymic synthesis of GDP-[5- ^3H]D-Ara

(a) $[5\text{-}^3\text{H}]\text{D-Ara}$

$[6\text{-}^3\text{H}]\text{D-GlcN}$ (300 μCi) was reacted with 17 mM ninhydrin in 300 μl of 25 mM citrate/NaOH, pH 4.7 (100 $^\circ\text{C}$, 40 min) [15]. The reaction mixture was desalted by passage through AG50X-12 (H^+) over AG3-X4 (OH^-) (200 μl of each) and $[5\text{-}^3\text{H}]\text{D-Ara}$ in the neutral eluate was purified by cellulose TLC. Two main radioactive products were formed: $[5\text{-}^3\text{H}]\text{D-Ara}$ and a product that co-migrated with ribose. A time-course experiment indicated that all of the $[6\text{-}^3\text{H}]\text{D-GlcN}$ had reacted by 40 min and that longer incubations resulted in the formation of unwanted degradation products. Purified $[5\text{-}^3\text{H}]\text{D-Ara}$, which was obtained in an overall yield of 40%, was stored at -20°C as a $1\ \mu\text{Ci}\cdot\mu\text{l}^{-1}$ solution in 90% ethanol. This product co-migrated with authentic D-Ara on both cellulose TLC and Dionex HPAEC.

(b) $[5\text{-}^3\text{H}]\text{D-Ara-1-PO}_4$

C. fasciculata D-arabinose-1-kinase activity eluted from the Superdex-200 column was utilized for the regioselective phosphorylation of $[5\text{-}^3\text{H}]\text{D-Ara}$ at position 1. The reaction was carried out in 50 mM $\text{NaH}_2\text{PO}_4/\text{NaOH}$, pH 6.7, 10 mM MgSO_4 , 100 mM KCl, 600 μM ATP containing approximately 6 μM $[5\text{-}^3\text{H}]\text{D-Ara}$ ($63\ \text{nCi}\cdot\mu\text{l}^{-1}$) at 30°C . Under these conditions, 52%, 88% and 90% of the label was converted into D-Ara-1- PO_4 after 5 min, 30 min and 60 min respectively. At time 60 min, the reaction mixture was boiled for 2 min, centrifuged, diluted 20 times with water, loaded on to a SAX-HPLC column equilibrated in 5 mM ammonium acetate, and eluted with a gradient of ammonium acetate (5–500 mM over 20 min). The $[5\text{-}^3\text{H}]\text{D-Ara-1-PO}_4$ in the retained fractions was freeze-dried twice and converted into its triethylammonium salt by passage through a column of Chelex-100 (Na^+) (100 μl) over AG50-X12 (H^+) (400 μl), followed by neutralization with triethylamine. As expected for $[5\text{-}^3\text{H}]\text{D-Ara-1-PO}_4$, this compound was entirely converted into neutral $[5\text{-}^3\text{H}]\text{D-Ara}$ upon mild acid hydrolysis.

(c) GDP-[5- ^3H]D-Ara

GDP-[5- ^3H]D-Ara was synthesized essentially as described previously [16]. $[5\text{-}^3\text{H}]\text{D-Ara-1-PO}_4$ (3 μCi) was taken to dryness in a 10 ml glass flask. Tri-*N*-octylamine (1 μl) was added and the mixture was co-evaporated five times with 2 ml of dry pyridine. GMP-morpholidate (3 mg in 2 ml of dry pyridine) was added and the mixture was co-evaporated twice more with dry pyridine. The reaction mixture was finally dissolved in 500 μl of dry pyridine and left for 6 h at 60°C , followed by 48 h at room temperature. Pyridine was removed by evaporation. The residue was dissolved in water and the GDP-[5- ^3H]D-Ara product was purified by SAX-HPLC (in KH_2PO_4 buffer; see above) in a yield of 60%. The fraction of interest was desalted by two successive steps of gel-permeation chromatography on Sephadex-G10 (1 cm $\times$ 50 cm) equilibrated in water. Under these conditions, GDP-[5- ^3H]D-Ara eluted in the void volume of the column. Synthetic GDP-[5- ^3H]D-Ara co-migrated with authentic GDP- α -D-Ara_p on SAX-HPLC and yielded neutral $[5\text{-}^3\text{H}]\text{D-Ara}$ after mild acid hydrolysis.

RESULTS

D-Ara metabolites can be synthesized from glucose

In a previous study using *L. major*, we noticed that commercial $[2\text{-}^3\text{H}]\text{D-Glc}$ is contaminated with $[^3\text{H}]\text{D-Ara}$ and we suggested that this might explain the facile labelling of D-Ara-1- PO_4 , GDP-

Figure 1 Labelling of GDP-d-Ara with radiolabelled d-Ara and d-Glc

C. fasciculata was labelled with $[^3\text{H}]\text{d-Ara}$ (a and b) or with purified $[2\text{-}^3\text{H}]\text{d-Glc}$ (c and d), in the presence (b and d) or absence (a and c) of 2 mM d-Ara. The labelling medium also contained 2 mM d-ribose. Nucleotides in the soluble extract were separated by SAX-HPLC and the eluted radioactivity was detected by liquid scintillation counting. Nucleotides and sugar nucleotides were detected by their UV absorbance at 262 nm and their elution positions are indicated as follows: peak 1, UDP-HexNAc; peak 2, UDP-Hex; peak 3, GDP-Hex; peak 4, UDP/NADP; peak 5, ADP; peak 6, GDP; peak 7, ATP.

d-Ara and LPG of *L. major* in the d-Ara residues [9]. In this study commercial $[^3\text{H}]\text{d-Glc}$ was fractionated into pure $[^3\text{H}]\text{d-Glc}$ and pure $[^3\text{H}]\text{d-Ara}$ by Dionex HPAEC and each of these radioactive monosaccharides was used to biosynthetically label *C. fasciculata* cells.

Two negatively charged water-soluble metabolites eluting at about 21 min and 43 min from the SAX-HPLC column were biosynthetically labelled by both $[^3\text{H}]\text{d-Ara}$ and $[2\text{-}^3\text{H}]\text{d-Glc}$ (Figures 1a and 1c) and co-migrated precisely with Ara-1- PO_4 and GDP-Ara from *L. major*. The compound eluting at 21 min yielded a single neutral monosaccharide upon mild acid hydrolysis or alkaline phosphatase treatment, that co-migrated with authentic Ara on both Dionex HPAEC and cellulose TLC (Table 1). The Ara residue could be reduced to arabinitol only if the acid hydrolysis was performed prior to the reduction, suggesting that the phosphate group is located on the anomeric carbon of the Ara residue. Taken together, these results are

consistent with the peak eluting at 21 min being Ara-1- PO_4 (Table 1). The metabolite eluting at 43 min was resistant to alkaline phosphatase digestion but yielded radioactive Ara after mild acid hydrolysis. A second radiolabelled, negatively charged hydrolysis product was produced from the $[2\text{-}^3\text{H}]\text{d-Glc}$ -labelled compound that co-migrated with authentic GDP on SAX-HPLC (Table 2). {The nature of the base has a strong influence on the retention time of nucleotides and sugar nucleotides on SAX-HPLC, as exemplified by the series UDP, CDP, ADP, GDP [9] or UDP-Glc, CDP-Glc, ADP-Glc, GDP-Glc (results not shown). If the Ara residue was linked to a nucleoside diphosphate other than GDP, it would be expected to elute significantly earlier, before GDP-Man.} Taken together, these data strongly suggest that the compound eluting at 43 min is GDP-Ara.

Cells labelled with pure $[^3\text{H}]\text{d-Ara}$ produced only two negatively charged water-soluble metabolites, Ara-1- PO_4 and GDP-

Table 1 Characterization of metabolically labelled Ara-1-PO₄, GDP-Ara and UDP-Hex

C. fasciculata cells were biosynthetically labelled with either purified [2-³H]D-Glc or purified [³H]D-Ara. A soluble metabolite fraction was prepared and analysed by SAX-HPLC. Radioactivity co-migrating with Ara-1-PO₄, GDP-Ara and UDP-Hex was collected and submitted to chemical and enzymic treatments. Degradation products were identified by co-migration with unlabelled standards on SAX-HPLC (for negatively charged compounds) and Dionex HPAEC (for neutral monosaccharides). UDP-Hex was not labelled with [³H]D-Ara. Abbreviations: H⁺, mild acid hydrolysis; H⁺/NaBH₄, mild acid hydrolysis followed by reduction with NaBH₄; NaBH₄/H⁺, NaBH₄ reduction followed by mild acid hydrolysis.

Metabolically labelled compound	Biosynthetic label used	Labelled degradation products			
		H ⁺	Alkaline phosphatase	H ⁺ /NaBH ₄	NaBH ₄ /H ⁺
Ara-1-PO ₄	[2- ³ H]D-Glc	Ara			
	[³ H]D-Ara	Ara	Ara	Arabinitol	Ara
GDP-Ara	[2- ³ H]D-Glc	Ara (96%)	GDP-Ara		
		GDP (4%)			
	[³ H]D-Ara	Ara			
UDP-Hex	[2- ³ H]D-Glc	Glc (67%)	UDP-Hex		
		Gal (28%)			
		UDP (15%)			

Table 2 Labelling of the glycosyl moiety of UDP-Hex and GDP-Ara with [³H]D-Glc

C. fasciculata were biosynthetically labelled with purified [1-³H]D-Glc, [2-³H]D-Glc and [6-³H]D-Glc, in the absence of D-ribose. A soluble extract was prepared and analysed by SAX-HPLC. Fractions corresponding to UDP-Hex and GDP-Ara were submitted to mild acid hydrolysis and the released labelled neutral monosaccharides were identified by co-migration with unlabelled standards on Dionex HPAEC. Radioactivity was quantified by liquid scintillation counting.

Label	UDP-Hex		GDP-Ara
	Glc (c.p.m.)	Gal (c.p.m.)	Ara (c.p.m.)
[1- ³ H]D-Glc	5900	1300	< 20
[2- ³ H]D-Glc	2300	900	820
[6- ³ H]D-Glc	5500	1500	480

Ara (Figure 1a). The absence of any other radioactive metabolites suggests that *C. fasciculata* is able to take up D-Ara and to incorporate it directly into D-Ara-1-PO₄ via the action of a D-arabino-1-kinase. As expected, the inclusion of unlabelled D-Ara in the labelling medium abolished the labelling of Ara-1-PO₄ and GDP-Ara (Figure 1b) whereas the inclusion of L-Ara had no effect (results not shown).

Cells labelled with pure [2-³H]D-Glc produced a wide variety of negatively charged water-soluble metabolites (Figure 1c) such as nucleoside-diphosphates and triphosphates, sugar nucleotides and sugar-phosphates, including Ara-1-PO₄ and GDP-Ara. These results suggest that *C. fasciculata* can convert D-Glc into D-Ara for the production of D-Ara-1-PO₄ and GDP-Ara. This conclusion was further supported by the observation that the inclusion of D-Ara in the labelling medium selectively inhibited the production of labelled Ara-1-PO₄ and GDP-Ara (Figure 1d).

The conversion of D-Glc into D-Ara by *C. fasciculata* is via the loss of the C-1 carbon atom

C. fasciculata was labelled with purified [1-³H]D-Glc, [2-³H]D-Glc or [6-³H]D-Glc and the cell extracts were separated by SAX-HPLC. The UV-absorbing peaks corresponding to UDP-Hex and GDP-Ara were subjected to mild acid hydrolysis and their radioactive neutral products were analysed by Dionex HPAEC.

All three labels were incorporated into the hexose (Glc and Gal) residues of the UDP-Hex fraction, but only [6-³H]D-Glc and [2-³H]D-Glc were incorporated into the Ara residue of GDP-Ara (Table 2). These data suggest that the conversion of D-Glc into D-Ara by *C. fasciculata* involves the loss of the C-1 carbon atom of D-Glc. The possibility that D-GlcN could be an intermediate in this conversion was ruled out because purified [6-³H]D-GlcN was unable to label GDP-Ara, despite its very efficient incorporation into UDP-HexNAC (results not shown).

Ara-1-PO₄ and GDP-Ara are used for LAG biosynthesis in *C. fasciculata*

C. fasciculata expresses an arabinogalactan, composed of D-Ara_p and β-D-Gal_p [2,3], linked to a glycosylphosphatidylinositol (GPI)-like membrane anchor (P. Schneider, K. G. Milne, A. Treumann, M. J. McConville, N. Zitzmann and M. A. J. Ferguson, unpublished work). We refer to this glycoconjugate as lipoarabinogalactan or LAG.

A hydrophobic molecule that was metabolically labelled with [5-³H]D-Ara was extracted from delipidated cell pellets and purified by octyl-Sepharose chromatography. This molecule behaved like authentic LAG. In particular, it was sensitive to nitrous acid deamination, which is diagnostic of a GPI anchor. Strong acid hydrolysis of this material yielded a single-labelled monosaccharide that co-migrated with Ara on Dionex HPAEC. Ara residues in 1-2 glycosidic linkage are known to be acid-labile [17]. Indeed, most (90%) of the radiolabelled Ara was released by mild trifluoroacetic acid hydrolysis. These results are consistent with the labelled hydrophobic material being LAG.

When cells were labelled continuously with [5-³H]D-Ara, Ara-1-PO₄ was the first metabolite to be labelled (reaching steady-state labelling within 1 min), followed by GDP-Ara and LAG (Figures 2a, 2b and 2c). When the [5-³H]D-Ara label was chased, by the addition of non-radioactive D-Ara after 1 min of labelling, the levels of radioactive Ara-1-PO₄ and GDP-Ara were rapidly depleted and the rate of incorporation of [5-³H]D-Ara into LAG was greatly reduced (Figures 2a, 2b and 2c). These data are consistent with the conversion of Ara-1-PO₄ into GDP-Ara, and the use of GDP-Ara as the arabinosyl donor for LAG biosynthesis. No apolar lipid was labelled with [5-³H]D-Ara, making it very unlikely that dolichol phosphate-type activated sugar

Figure 2 *In vivo* pulse-chase labelling and precursor-product relationships between D-Ara-1-PO₄, GDP-D-Ara and lipoarabinogalactan

C. fasciculata was labelled with [5-³H]-D-Ara (●). After 60 s (arrow), part of the cell suspension was adjusted to 5 mM with respect to unlabelled D-Ara (○). Aliquots were removed at the indicated times and labelling was stopped by extracting the cells with cold chloroform/methanol/water (see the Materials and methods section). The soluble nucleotide fractions, containing Ara-1-PO₄ (a) and GDP-D-Ara (b), were analysed by SAX-HPLC as shown in Figure 1. Lipoarabinogalactan (LAG, c) was extracted from the delipidated cell pellet with 9% 1-butanol and purified by octyl-Sepharose chromatography.

donors are involved in the transfer of arabinosyl residues to LAG (results not shown).

The identification of a D-arabino-1-kinase activity

A cytosolic fraction was prepared from *C. fasciculata* and was found to be able to convert [5-³H]D-Ara (in the presence of ATP,

Figure 3 Enzymic assay and donor substrate requirement for D-arabino-1-kinase activity of *C. fasciculata*

Partially purified D-arabino-1-kinase activity was incubated for the indicated times with [5-³H]D-Ara and 2 mM ATP in the presence of 10 mM MgSO₄ or 20 mM EDTA (a). The effects of different nucleotide triphosphates (all at 2 mM, in the presence of 10 mM MgSO₄) were also tested in a 30 min reaction (b). Reactions were stopped on ice by the addition of 1-propanol and the products separated by chromatography on silica 60 HPTLC. Radioactivity was detected by fluorography after spraying with En³Hance. The migration positions of D-Ara and Ara-1-PO₄ are indicated on the left-hand side of the chromatogram.

Mg²⁺ and the phosphatase inhibitor NaF) into a product with the same retention time on SAX-HPLC as authentic D-Ara-1-PO₄. As expected for a sugar-1-phosphate, mild acid hydrolysis rendered this product neutral (results not shown). The cytosolic fraction was subjected to gel-permeation chromatography on a Superdex-200 column. The D-arabino-1-kinase activity was included by the column and eluted with an apparent molecular mass of approximately 600 kDa. Phosphatase activities eluted with apparent molecular masses of approximately 100 kDa, 65 kDa and 32 kDa and were completely removed from the D-arabino-1-kinase activity by this single partial purification step. When ATP was incubated alone or with the fractions containing the D-arabino-1-kinase activity for up to 1 h at 37 °C, the elution profiles after SAX-HPLC analysis were virtually identical, with more than 95% of the UV-absorbing material eluting as ATP (results not shown). This indicates that the partially purified D-arabino-1-kinase is free of ATP-degrading activities in the absence of D-Ara.

Characterization of the D-arabino-1-kinase activity

The enzyme activity was dependent on the presence of Mg²⁺ and was abolished by the inclusion of 20 mM EDTA (Figure 3a) or by replacing ATP with GTP, CTP or UTP (Figure 3b). These data indicate that MgATP is the donor substrate for the D-arabino-1-kinase. The enzyme was most active in the range pH 6.0 to pH 8.5 and showed a pH optimum of about pH 7.2 (results not shown).

Kinetic analysis of the D-arabino-1-kinase activity suggested a *K_m* of 24 μM for D-Ara and 1.7 mM for ATP (Figure 4). Substrate inhibition at concentrations of D-Ara above 100 μM was observed (Figure 4a).

In order to investigate the acceptor substrate specificity of the

Figure 4 Kinetic analysis of the D-arabino-1-kinase activity

Initial velocities in the presence of [5-³H]D-Ara, 10 mM MgSO₄ and various amounts of ATP and unlabelled D-Ara were determined at pH 6.7 and at 30 °C, as described in the Materials and methods section. (a) Dependence of V_0 on D-Ara concentration at two concentrations of ATP (0.6 mM, ■; 1.2 mM, □). (b) and (c) Lineweaver-Burk representation of the data obtained with concentrations ranging from 5 to 100 μM for D-Ara (b: 5 μM, ●; 6.7 μM, ○; 10 μM, ■; 20 μM, □; 40 μM, ▲; 100 μM, △) and 0.3 to 2.5 mM for ATP (c: 0.3 mM, ●; 0.4 mM, ○; 0.6 mM, ■; 1.2 mM, □; 2.5 mM, ▲). Replots of the slopes and intercepts obtained in (b) and (c) are shown in (d) and (e), and (f) and (g) respectively.

enzyme a number of monosaccharides were assayed for their ability to inhibit the conversion of [5-³H]D-Ara into [5-³H]D-Ara-1-PO₄ (Figure 5). In this assay non-radioactive D-Ara gave 50% inhibition (IC_{50}) at 300 μM whereas the optical isomer L-Ara was not inhibitory at up to 30 mM. The epimers of D-Ara at positions 2 and 3 (D-Rib and D-lyxose respectively) were poor inhibitors (with IC_{50} values of 50 mM and 80 mM respectively) whereas the epimer at position 4 (L-xylose) was hardly inhibitory at concentrations as high as 100 mM. The D-Ara analogue L-Gal was a reasonable inhibitor (IC_{50} value of 12 mM) but the 2, 3, and 4 epimers of L-Gal (L-talose, L-gulose and L-glucose respectively) were non-inhibitory. D-Glc, D-Man, D-Gal, D-GlcN and D-GlcNAc at 10 mM were also non-inhibitory (results not shown). Taken together, these results suggest that the orientation of the 4-hydroxyl group of D-Ara is essential for substrate recognition while the orientations of the 2- and 3-hydroxyl groups are also important. The inhibitory characteristics of L-Gal (IC_{50} 12 mM) and L-Fuc (IC_{50} 50 μM) suggest that an equatorial hydroxymethyl

Figure 5 Inhibitory effect of monosaccharides on the phosphorylation of [5-³H]D-Ara by D-arabino-1-kinase activity

Assays were performed for 30 min under conditions where more than 95% of [5-³H]D-Ara was converted into [5-³H]D-Ara-1-PO₄. Unlabelled pentoses [D-Ara, L-Ara, D-ribose (D-Rib), D-lyxose (D-Lyx) and L-xylose (L-Xyl)], hexoses [L-Gal, L-Glc, L-talose (L-Tal), L-gulose (L-Gul)] and 6-deoxyhexose [L-fucose (L-Fuc)] were added at concentrations ranging from 30 μM to 100 mM. The reaction products were separated by HPTLC and the areas where D-Ara and D-Ara-1-PO₄ migrated were scraped and counted by liquid scintillation.

group attached to C-5 of D-Ara (in L-Gal) can be tolerated by the enzyme and that a methyl group at the same position (in L-Fuc) actually enhances substrate recognition.

The chemico-enzymic synthesis of GDP-[5-³H]D-Ara

The synthesis scheme of GDP-[5-³H]D-Ara is shown in Figure 6. [6-³H]D-Glucosamine was converted into [5-³H]D-Ara by reaction with ninhydrin. A time-course of this reaction showed that the optimum yield (40%) was achieved after 40 min (results not shown). The [5-³H]D-Ara was purified by cellulose-TLC and phosphorylated (in a yield of 90%) using the partially purified D-arabino-1-kinase activity described above. The [5-³H]D-Ara-1-PO₄ product was purified by SAX-HPLC, converted into its triethylammonium salt and reacted with a large excess of GMP-morpholidate in dry pyridine. The GDP-[5-³H]D-Ara, produced in 60% yield, was purified by SAX-HPLC, where it co-eluted with authentic GDP-α-D-Ara_p, and was desalted by Sephadex-G10 chromatography.

DISCUSSION

GDP-α-D-Ara_p acts as the activated donor of D-Ara_p residues in *L. major* [9], *C. fasciculata* (this study) and, probably, in all trypanosomatids that utilize this particular sugar. The proposed pathway of GDP-D-Ara_p biosynthesis in *C. fasciculata* is shown in Figure 7. This pathway appears to be conserved in *L. major* and *C. fasciculata* because both organisms metabolize labelled D-

Figure 6 Scheme for the chemico-enzymic synthesis of GDP-[^3H]D-Ara

Abbreviations: AG50, Dowex AG 50-X12 (H^+); AG3, Dowex AG 3-X4 (OH^-); TEA, triethylamine.

Figure 7 Proposed biosynthetic origin of D-Ara in *C. fasciculata* lipoarabinogalactan

D-Ara can either be taken up by the cell or can be derived from D-Glc with loss of the carbon C-1. It is then phosphorylated by an MgATP-dependent D-arabino-1-kinase. GDP-D-Ara is probably formed from D-Ara-1- PO_4 and GTP by a glycosyl-1-phosphate nucleotidyltransferase (D-Ara-1- PO_4 :GTP transferase) reaction which presumably occurs in the cytosol. A putative transporter then translocates GDP-D-Ara to the site of lipoarabinogalactan (LAG) synthesis, which is most probably the endoplasmic reticulum (ER) and/or the Golgi apparatus. GDP-D-Ara is utilized by a glycosyltransferase for the arabinosylation of the repeating β -Gal residues of the lipoarabinogalactan backbone. Abbreviation: PM, plasma membrane.

Ara in a similar way, although with different kinetics ([9]; this study). These parasites are able to internalize D-Ara directly from the extracellular medium. However, it is unlikely that they will find sufficient amounts of free D-Ara in their natural environments (i.e. in the lumen of insect guts) and, presumably, they must be able to synthesize it from another substrate. In this respect, it is significant that *C. fasciculata* can produce D-Ara from D-Glc.

The conversion of D-Glc into D-Ara appears to involve the loss of the C-1 carbon atom of D-Glc, although the precise route of this conversion is not clear. One plausible route would involve the conversion of D-Glc into D-ribulose-5- PO_4 , followed by dephosphorylation and epimerization to D-Ara. However, we consider this route unlikely because there is some evidence from biosynthetic labelling experiments that ribulose-5- PO_4 is not an intermediate. (The labelling of nucleoside di- and tri-phosphates (and other nucleotides) with [$2-^3\text{H}$]D-Glc is reduced by the inclusion of D-Rib in the labelling medium [9], suggesting that [$2-^3\text{H}$]D-Glc label can be incorporated into the D-Rib moieties of the corresponding nucleosides. In the absence of D-Rib, the labelling of nucleoside di- and tri-phosphates with [$1-^3\text{H}$]D-Glc is comparable with that with [$2-^3\text{H}$]D-Glc and [$6-^3\text{H}$]D-Glc (results not shown), suggesting that [$1-^3\text{H}$]D-Glc can also be converted into [^3H]D-Rib. This could occur if [$1-^3\text{H}$]D-Glc is converted into [$3-^3\text{H}$]glyceraldehyde-3- PO_4 by glycolysis, then into [$5-^3\text{H}$]D-xylulose-5- PO_4 by the action of transketolase and finally into [$5-^3\text{H}$]D-Rib-5- PO_4 via [$5-^3\text{H}$]D-ribulose-5- PO_4 . Since the D-Ara residue of GDP-D-Ara cannot be biosynthetically labelled with [$1-^3\text{H}$]D-Glc (Table 2), we suggest that D-ribulose-5- PO_4 is not an intermediate in the conversion of D-Glc into D-Ara.) A route via D-GlcN, analogous to the reaction of D-GlcN with ninhydrin, can be ruled out since [$6-^3\text{H}$]D-GlcN does not act as a biosynthetic precursor for GDP-D-Ara. An alternative route could be the direct conversion of D-Glc into D-Ara via an unknown enzyme; however, there is currently no direct evidence for this.

The D-arabino-kinase activity of *C. fasciculata* consistently yielded the product Ara-1- PO_4 , even after partial purification by size-exclusion chromatography and anion-exchange chromatographies (results not shown). This suggests that the activity is a regioselective D-arabino-1-kinase, rather than the product of the combined actions of a kinase and a phosphomutase (cf. Glc $\rightarrow$ Glc-6- $\text{PO}_4 \rightarrow$ Glc-1- PO_4). Numerous other monosaccharides (i.e. D-Gal, D-glucuronic acid, D-galacturonic acid, D-xylose, L-Ara and L-Fuc) are also substrates for corresponding 1-kinase activities which catalyse the first step of sugar nucleotide biosynthesis in the so-called salvage pathway of monosaccharides [18]. However, given the large apparent molecular mass of the arabino-1-kinase activity (600 kDa) the possibility that this enzyme is part of a multi-enzyme complex can not be excluded.

The pulse-chase experiment (Figure 2) indicates that in *C. fasciculata* the radiolabelled D-Ara is sequentially incorporated into D-Ara-1- PO_4 and GDP-D-Ara before transfer to the LAG acceptor molecule. This sequence of events suggests that GDP-Ara may be formed by the reaction of D-Ara-1- PO_4 with GTP, catalysed by a specific glycosyl-1-phosphate nucleotidyltransferase (D-Ara-1- PO_4 :GTP transferase), and that the formation of GDP-Ara by the modification of another sugar nucleotide (cf. UDP-Glc $\rightarrow$ UDP-Gal) seems unlikely. This is in contrast to the situation in mycobacteria where the D-Ara, residues, found in the cell wall polysaccharides, cannot be derived directly from D-Ara. In this case, the activated donor (β -D-Ara $_7$ -monophosphodecaprenol) is produced by the epimerization of a β -D-ribofuranose-monophosphodecaprenol precursor [19].

The D-arabino-1-kinase activity displayed a K_m for D-Ara of

24 μM and an absolute requirement for MgATP. The K_m for MgATP (about 1.7 mM) is quite high and suggests that the metabolic state of the cell, and hence the cytosolic ATP level, might modulate the rate of D-Ara-1- PO_4 synthesis and, in turn, the level of LAG expression. This high K_m for ATP also explains why none of the available ATP-agarose affinity resins are capable of retaining the enzyme (results not shown). Competition studies using a variety of monosaccharides indicated that the D-arabino-1-kinase is rather selective for the orientation of all of the hydroxyl groups of D-Ara, whereas it can accommodate an equatorial substituent (methyl or hydroxymethyl groups; as in L-Fuc and L-Gal, respectively) at position 5 of D-Ara. Indeed, L-Fuc appears to out-compete D-Ara for binding to the enzyme active site, although we have not determined whether or not the enzyme phosphorylates L-Fuc. As *C. fasciculata* and *L. major* do not make use of L-Fuc, this lack of specificity between D-Ara and L-Fuc should not hinder these parasites.

In this study, we have used the D-arabino-1-kinase activity for the regioselective and almost quantitative phosphorylation of radioactive D-Ara. The latter was made by reacting commercial [6- ^3H]D-glucosamine with ninhydrin. The formation of the appropriate glycosyl-1-phosphate is the most difficult step in the chemical synthesis of sugar nucleotides [18] and these reactions are not usually performed on a high-specific-activity radiochemical scale. Thus the D-arabino-1-kinase activity was extremely convenient in this synthesis. The resulting [5- ^3H]D-Ara-1- PO_4 was converted into GDP-[^3H]Ara in reasonable yield by reaction with GMP-morpholidate [16,18].

The availability of [^3H]D-Ara has already proved to be useful in the assay, characterization and partial purification of the D-arabino-1-kinase from *C. fasciculata*. The chemico-enzymic synthesis of [^3H]D-Ara-1- PO_4 and GDP-[^3H]D-Ara should also prove to be useful in the development of assays for the putative D-Ara-1- PO_4 :GTP transferase and the GDP-Ara:acceptor glycolipid (1-2)-arabinopyranosyltransferases of *C. fasciculata* and *L. major*. The assay, characterization and purification of all of these enzymes are biologically relevant. In *L. major*, the use of D-Ara in LPG biosynthesis is under a developmental control [6]. This could, in principle, occur at any level of the synthesis, transport and/or use of any precursor of the D-Ara_p residues, including D-Ara-1- PO_4 and GDP-D-Ara (Figure 7). Thus the establishment

of the pathway of GDP-Ara biosynthesis, and the chemico-enzymic synthesis of the intermediates, will allow us to address the molecular basis of D-Ara_p regulation in *L. major*.

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