Molecular Cloning of the *Leishmania major* UDP-glucose Pyrophosphorylase, Functional Characterization, and Ligand Binding Analyses Using NMR Spectroscopy

Anne-Christin Lamerz, Thomas Haselhorst, Anne K. Bergfeld, Mark von Itzstein, and Rita Gerardy-Schahn

From the Zelluläre Chemie, Zentrum Biochemie, Medizinische Hochschule Hannover, Carl-Neuberg-Strasse 1, 30625 Hannover, Germany and the Institute for Glycomics, Griffith University (Gold Coast Campus), PMB 50 Gold Coast Mail Centre, 9726, Australia

The dense glycocalyx surrounding the protozoan parasite *Leishmania* is an essential virulence factor. It protects the parasite from hostile environments in the sandfly vector and mammalian host and supports steps of development and invasion. Therefore, new therapeutic concepts concentrate on disturbing glycocalyx biosynthesis. Deletion of genes involved in the metabolism of galactose and mannose have been shown to drastically reduce *Leishmania* virulence. Here we report the identification of *Leishmania major* UDP-glucose pyrophosphorylase (UGP). UGP catalyzes the formation of UDP-glucose from glucose-1-phosphate and UTP. This activation step enables glucose to enter metabolic pathways and is crucial for the activation of galactose. UDP-galactose is made from UDP-glucose by nucleotide-donor transfer to galactose-1-phosphate or by epimerization of the glucose moiety. Isolated in a complementation cloning approach, the activity of *L. major* UGP was proven in vitro. Moreover, purified protein was used to investigate enzyme kinetics, quaternary organization, and binding of ligands. Whereas sequestration by oligomerization is a known regulatory mechanism for eukaryotic UGPs, the recombinant as well as native *L. major* UGP migrated as monomer in size exclusion chromatography and in accord with this showed simple Michaelis-Menten kinetics toward all substrates. In saturation transfer difference (STD)-NMR studies, we clearly demonstrated that the molecular geometry at position 4 of glucose is responsible for substrate specificity. Furthermore, the γ-phosphate group of UTP is essential for binding and for induction of the open conformation, which then allows entry of glucose-1-phosphate. Our data provide the first direct proof for the ordered bi-bi mechanism suggested in earlier studies.

*Leishmania* are protozoan parasites that cause severe diseases in human and animals. Depending on the *Leishmania* strain, disease manifestations can range from self-healing cutaneous lesions to fatal visceral forms. Based on records of the World Health Organization, 12 million people are infected worldwide, but no vaccine or specific drug is available to prevent or treat *Leishmaniasis*. 

*Leishmania* are covered by a dense glycoconjugate layer, the glycocalyx, which helps the parasite to withstand hostile environments in the sandfly vector and mammalian host. Moreover, structural changes in glycocalyx components accompany the life cycle of the parasites and are crucial for the transmission of the parasite (2, 3).

A major component of the glycoconjugates is galactose, which is dynamically added in the Golgi apparatus. Both transport of galactose to the Golgi lumen and insertion into glycoconjugates depend on the synthesis of the nucleotide sugar UDP-Gal in the cytoplasm of the cell. Whereas synthesis of most nucleotide sugars is catalyzed by pyrophosphorylases using the respective nucleotide triphosphate as substrate (for a review, see Ref. 4), activation of galactose is achieved by nucleotide exchange between UDP-Glc and galactose 1-phosphate (Gal-1-P). Alternatively, epimerization of sugar position 4 in UDP-Glc leads to UDP-Gal (for a review, see Ref. 5). The two ways of galactose activation are shown in Fig. 1. The scheme in addition highlights the fact that UDP-Gal synthesis essentially depends on the availability of UDP-Glc.

Knowing the importance of galactose-containing glycoconjugates for *Leishmania major* virulence (6, 7), the aim of this study was to identify potential *L. major* UDP-Gal transporters (UGTs). In this context, it is important to mention that galactose in *Leishmania* exists in two conformers, as pyranose and furanose (8–10). Whereas galactopyranose is ubiquitously present in the animal kingdom (referred to as galactose), galactofuranose is unknown (11) but is believed to be an essential virulence (sometimes even viability) factor for various pathogens (12). Because the formation of UDP-Gal and the synthesis of UDP-galactofuranose from UDP-Gal by the enzyme UDP-galactopyranose mutase (13, 14) proceeds in the cytoplasm, Golgi transport systems for both UDP-Gal conformers should exist in *L. major*.

To identify these transporters, a heterologous complementation cloning approach was initiated using an *L. major* cDNA library and UGT-negative LeC8 cells as hosts. Different from the expected results, we identified the *L. major* UDP-glucose pyrophosphorylase (UGP; EC 2.7.7.9), an enzyme located upstream of the Golgi transporters. As shown in Fig. 1, UGP catalyzes the synthesis of UDP-Glc from glucose-1-phosphate (Glc-1-P) and UTP and thus provides an essential substrate for UDP-Gal formation. Moreover, UGP activity is required for the entry of galactose into glycolysis and thus for its availability in energy production. Finally, UDP-Glc itself is transported into the Golgi and used by glucosyltransferases that dynamically decorate glycoconjugates.

The strategically important position of the newly identified gene prompted us to carefully investigate its catalytic properties and thus eventually evaluate a novel target for future therapeutic approaches.

The nucleotide sequence(s) reported in this paper has been submitted to the GenBank with accession number(s) DQ328944. 

□ 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.

© 2006 by The American Society for Biochemistry and Molecular Biology, Inc. Printed in the U.S.A.

1 Supported by an Australian Federation Fellowship of the Australian Research Council and by the National Health and Medical Research Council.

2 Supported by Deutsche Forschungsgemeinschaft Grant Ge 801/4-1/2 and the Fonds der Chemischen Industrie. To whom correspondence should be addressed. Tel.: 49-511-532-9802; Fax: 49-511-532-8801; E-mail: gerardy-schahn.rita@mh-hannover.de.

3 The abbreviations used are: Gal-1-P, galactose 1-phosphate; CHO, Chinese hamster ovary; UGT, UDP-galactose transporter; UDP-Glc, UDP-glucose pyrophosphorylase; STD, saturation transfer difference; Glc-1-P, glucose 1-phosphate; Rib, ribose; Ur, uridine.
Experimental Procedures

Cells and Reagents—The Chinese hamster ovary (CHO) cell line Lec8 (Stanley) was obtained from ATCC and cultured in a minimum essential medium (Invitrogen) supplemented with 5% fetal calf serum (Invitrogen). A cDNA library was constructed from the promastigote form of L. major in the eukaryotic expression vector pCMV-Script (Stratagene). The rat gluturonyltransferase cDNA clone in the vector pEFBS (16) was used in cotransfection experiments (pEFBS-GlcAT). The rat monoclonal antibody L2-412 recognizing the HNK-1 epitope was kindly provided by M. Schachner (Hamburg, Germany), and goat anti-rat alkaline phosphatase was obtained from Jackson ImmunoResearch. Plasmids pOTB7-hUGP1 and pCMV-hUGP2 containing cDNAs of the human UGP isoforms a and b, respectively, were kindly provided by the RZPD Deutsches Ressourcenzentrum für Genomforschung GmbH (library number 958 clone IMAGp958H18235Q2 and library number 998 clone IMAGp998K047340Q3, respectively). pET expression vectors and Escherichia coli BL21(DE3) were purchased from Novagen. The E. coli DEV6 galU mutant strain (17) was obtained from the coli genetic stock collection at the E. coli genetic resource center, Yale University (New Haven, CT).

Complementation Cloning in CHO Lec8 Cells—Since isolation of a L. major UGT was the initial goal of this study, complementation cloning in CHO Lec8 cells was used as a strategy. For the heterologous complementation cloning approach, an L. major cDNA library was constructed from 5 μg of mRNA using the pCMV-ScriptXR cDNA library construction kit from Stratagene. The mRNA sample was purified from total RNA of the promastigote form of L. major (V121) with the RNeasy Midi kit (Qiagen). To enable sibling selection, the cDNA library was subdivided into 86 pools of 20,000 independent colonies. The plasmid DNA was transiently transfected into CHO Lec8 cells, and positive pools were identified by immunocytochemistry as described below. Of 22 tested pools, eight were able to complement Lec8 cells. The Me2SO stock corresponding to the positive pool 19 was selected and subdivided into pools of 1500 individual colonies. Plasmid DNA was isolated from subpools, and transfection and monitoring were repeated. After six rounds of sibling selection, a single cDNA clone (pACM1), which efficiently complemented Lec8 cells, was isolated.

For transient expression of human UGPs (isoforms a and b) in CHO Lec8 cells, the corresponding cDNAs were amplified from the plasmids pOTB7-hUGP1 and pCMV-hUGP2 with the primer pairs ACM 10 (CGCGGGATCCTCGAGATTGTACAAGATC/ACM 9 (ATATGGCCGTCGACTGTTCCAAGATGC) and ACM8 (CGCGGGATCCTCCAAGATGTCGTC/ACM9 (ATATGGCCGTCGACTGTTCCAAGATGC), respectively. Because the primers contained BamHI and NotI restriction sites (underlined), products could be digested with BamHII and NotI and, after gel purification (Qiagen), ligated into the corresponding restriction sites of pcDNA3-FLAG (19). The resulting plasmids pFL-hUGP and pFL-hUGP2 drove the expression of N-terminally FLAG-tagged proteins. For comparison, the L. major UGP was similarly amplified from cDNA library pool 19 using the primer pair ACM5 (CGCGGGATCCTCGAGATTGTACAAGATC/ACM6 (CGTTGCGGGCCTTTAGCCAACCATCGGGGATCACAAAC), containing BamHI and NotI restriction sites (underlined), respectively. After endonuclease digestion and gel purification, the DNA fragments were inserted into pcDNA3-FLAG as described above. The resulting vector was named pFL-LmUGP. Complementation by the hamster UGT (pcNDA3-FLAG-SO1) (19) served as a positive control. All constructs were transiently transfected into CHO Lec8 cells and analyzed by immunocytochemistry (see below).

The UDP-glucose Pyrophosphorylase of L. major

Transient Transfection and Immunocytochemistry—For transient transfections, 3 × 105 Lec8 cells were seeded in 6-well plates and 24 h later co-transfected with 0.5 μg of the respective cDNA plus 0.5 μg of the plasmid pEFBS-GlcAT, encoding the rat glucuronolyltransferase (16). Transfections were carried out with Lipofectamine (Invitrogen) and stopped after 6–8 h by medium exchange. Complementation was analyzed by immunocytochemistry with the monoclonal antibody L2-412 directed against the HNK-1 epitope (20). This methodology used to monitor complementation of Lec8 cells has been recently developed and approved in our laboratory (21). In brief, 48 h after transfection, CHO Lec8 cells were washed with Tris-buffered saline, fixed with 1.5% glutaraldehyde (10 min), blocked in 2% milk powder/Tris-buffered saline (30 min), and stained with the rat monoclonal antibody L2-412 for 1 h. After washing with Tris-buffered saline, bound monoclonal antibody L2-412 was detected with alkaline phosphatase-conjugated goat anti-rat antibody. Fast-Red (Sigma) was used as substrate.

Recombinant Expression of L. major UGP—To recombinantly express L. major UGP, the entire open reading frame was amplified with the primer pair ACM112 (CTGACTCCATATGGAAAGCACAT-GAACTCCTC) and ACM69 (CTTAGGGCCGCGCTGGTGCAC-GACTGCTGGC), containing NdeI and NotI restriction sites (underlined), respectively. The resulting PCR product was digested with NdeI and NotI, gel-purified, and ligated into the corresponding restriction sites of pET22b (Novagen). The resulting plasmid pET-UGP-His encodes the full-length, C-terminally His6-tagged L. major UGP. To generate the vector pET-UGP, allowing the expression of an untagged L. major UGP, the primer pair ACM112 and ACM7 (CTTAGGCGGGCCTGGATCCTACCTGTTGCTGACAGCTGGC) was used, and the PCR product was ligated into the Ndel and NotI restriction sites of pET22b. Integrity of the constructs was confirmed by sequencing. For expression, E. coli BL21(DE3) were transformed with the corresponding plasmid, and cells were grown in Luria-Bertani medium with 200 μg/ml carbenicillin at 15 °C to an A600 nm of 0.6. Then protein expression was induced by the addition of 1 mM isopropyl-1-thio-β-D-galactopyranoside, and the culture continued for 20 h. Thereafter, cells were harvested by centrifugation (4000 × g, 15 min, 4 °C), and pellets were washed once with phosphate-buffered saline and resuspended in buffer A (50 mM Tris/HCl, pH 7.8, 300 mM NaCl) if used for Ni2+-chelating chromatography or in buffer C (50 mM Tris/HCl, pH 8.0) if used for anion exchange chromatography, both supplemented with 40 μg/ml bestatin (Sigma), 4 μg/ml pepstatin (Sigma) and 1 mM phenylmethylsulfonyl fluoride (Roche Applied Science). Bacteria were lysed by sonification with a microtip (Branson Sonifer, 50% duty cycle, output control 5) using eight pulses of 30 s intermitted by breaks of 30 s. Cell debris was removed by centrifugation (20,000 × g, 15 min, 4 °C), and the soluble fractions were used for SDS-PAGE analyses or submitted to Ni2+-chelating or anion exchange chromatography to purify the recombinant protein.

Ni2+-chelating and Anion Exchange Chromatography—To purify the His6-tagged L. major UGP, soluble fractions of transformed bacteria, prepared as described above, were passed over a 5-ml HisTrap HP Ni2+-chelating column (Amersham Biosciences) according to the manufacturer’s instructions. The column was washed with 50 ml of buffer A (50 mM Tris/HCl, pH 7.8, 300 mM NaCl) followed by 30 ml of buffer A containing 40 mM imidazole. Bound protein was eluted with 300 mM imidazole in buffer A using a step gradient. For anion exchange chromatography, the soluble bacterial fraction was passed over a 1-ml Q-Sepharose FF column (Amersham Biosciences), and the column was washed with 10 ml of buffer C (50 mM Tris/HCl, pH 8.0). The protein was eluted using a linear gradient of 0–300 mM NaCl in 20 ml. The fractions containing L. major UGP were pooled and for buffer exchange
The UDP-glucose Pyrophosphorylase of *L. major*

passed over a HiPrep 26/10 column (Amersham Biosciences). Purified protein samples were stored at −80 °C in buffer B (50 mM Tris/HCl, pH 7.8, 10 mM MgCl₂).

**Preparation of *L. major* Cytosolic Fraction—Promastigote *L. major* (MHOM/SU/73/5ASKH) was grown in M199 medium supplemented with 5% fetal calf serum, 40 mM Hepes, pH 7.5, 0.1 mM adenine, 0.0005% hemin, 0.0002% biotin, and 50 units/ml penicillin/streptomycin to a density of 4–5 × 10⁷ cells/ml. The cells were harvested by centrifugation and washed twice in phosphate-buffered saline. Cells were lysed by freeze-thawing in buffer B (50 mM Tris/ HCl, pH 7.8, 10 mM MgCl₂), and the 100,000 × g supernatant was used for size exclusion chromatography.

**Size Exclusion Chromatography—** Size exclusion chromatography on a Superdex 200 HR 10/30 column (Amersham Biosciences) was used to determine the quaternary organization of the recombinant *L. major* UGP. Before sample loading, the column was equilibrated with 50 ml of buffer B (50 mM Tris/HCl, pH 7.8, 10 mM MgCl₂). In consecutive runs, the column was loaded with 100 µl of standard proteins (bovine serum albumin (10 mg/ml), bovine carbonic anhydrase (3 mg/ml), and yeast alcohol dehydrogenase (5 mg/ml)) as described by the manufacturer (protein standard kit; Sigma). Purified recombinant His₆-tagged or untagged *L. major* UGP (4 mg/ml) was loaded in the absence or presence of the substrates UTP (1 mM) and Glc-1-P (1 mM). Similarly, the *L. major* cytosolic fraction (3.5 mg/ml) was loaded to investigate the native protein.

**Complementation of E. coli DEV6 galU Mutant—** E. coli DEV6 galU mutants lack a functional UGP. This defect renders the bacterium unable to grow on galactose-containing MacConkey agar. For complementation assays, chemically competent E. coli DEV6 galU mutants were transformed with the plasmid pET-UGP-His or pET-UGP or the empty vector pET22b and grown on 2% Luria-Bertani agar supplemented with 100 µg/ml ampicillin. Colonies were streaked on 2% MacConkey agar plates. Colonies were streaked on 2% MacConkey agar plates. All of the signals in the STD-NMR spectrum (27) were acquired, and a WATERGATE sequence was applied to suppress the residual HDO signal. The total duration of the molecular ratio (protein/ligand) of 1:100. A total of 1024 scans/STD experiment were acquired, and a WATERGATE sequence was applied to suppress the residual HDO signal. Data acquisition and processing were performed using XWINNMR software (Bruker) on a Silicon Graphics O2 work station. Fourier transformation and base-line correction were performed for the ¹H NMR spectra recorded in 10-min time intervals over 1 h. Suppression of the residual HDO signal was achieved by presaturation with a weak radio frequency field for 1.5 s during the relaxation delay. Data acquisition and processing were performed with XWINNMR software (Bruker) on a Silicon Graphics O2 work station. Fourier transformation and base-line correction were performed for the ¹H NMR spectra recorded in 10 min. The protein was saturated on resonance at 0.7 ppm and off resonance at 40 ppm with a cascade of 40 selective Gaussian-shaped pulses of 50-ms duration with a 100-μs delay between each pulse in all STD-NMR experiments. The total duration of the saturation time was set to 2 s. For STD-NMR experiments, 0.6 µM recombinant UGP was used. All investigated ligands were added at a molecular ratio (protein/ligand) of 1:100. A total of 1024 scans/STD-NMR experiment were acquired, and a WATERGATE sequence was used to suppress the residual HDO signal. A lock spin filter with a strength of 5 kHz and a duration of 10 ms was applied to suppress protein background. Relative STD effects were calculated according to the equation: 

\[ A_{STD} = \frac{I_{STD} - I_{off}}{I_{off}} \]

where \( I_{STD} \) and \( I_{off} \) are the intensities of the signals in the STD-NMR spectrum (I_{STD}) with signal intensities of 50 mM Tris/HCl, pH 7.8, 10 mM MgCl₂, 1 mM 2-mercaptoethanol, 1 mM UTP, 2 mM Gal-1-P (Calbiochem), 1 mM NAD⁺, 0.05 units of UDP-glucose dehydrogenase and 0.05 units of UDP-galactose 4-epimerase (Calbiochem). All other assay conditions were identical. To determine \( K_m \) values, one substrate was present at a saturating concentration, whereas the concentration of the second substrate was varied. The initial rates were plotted against the substrate concentration, and the Excel Solver program (Microsoft) was used to approximate the data to the Michaelis-Menten equation (V = \( V_{max} ([S]/(S) + K_m) \)). In addition, Lineweaver-Burk plots were used to show the linear dependence and to calculate the \( K_m \) values.

**Antibody Preparation—** An antiserum against *L. major* UGP was prepared by subcutaneous injection of New Zealand rabbits with 500 µg of purified recombinant protein. For the first injection, protein was mixed with complete Freund’s adjuvant (Difco), followed by six injections at 6-week intervals using incomplete Freund’s adjuvant (Difco). Blood was collected 10 days after the last injection. The prepared serum specifically recognizes both recombinant and native forms of the *L. major* UGP.

**SDS-PAGE Analysis and Immunoblotting—** SDS-PAGE was performed according to Laemmli (24). Protein samples were separated on SDS-polyacrylamide gels composed of a 5% stacking gel and a 10% separating gel. Protein bands were visualized by silver staining (25). For Western blot analysis, proteins were transferred to nitrocellulose membranes (Schleicher & Schüll). His₆-tagged proteins were detected using the penta-His antibody (Qiagen) at a concentration of 1 µg/ml and goat anti-mouse Ig alkaline phosphatase-conjugate (Jackson ImmunoResearch). Untagged *L. major* UGP was detected using a 1/60,000 dilution of antiserum and alkaline phosphatase-conjugated goat anti-rabbit antibody (Jackson ImmunoResearch).

**DNA and Protein Sequence Analysis—** Homology searches were performed at the National Centre for Biotechnology Information using the program BLASTX2.2.3. For multiple alignments, the program MultAlin version 5.4.1 (26) was used.

**NMR—** All NMR experiments were performed on a Bruker Avance DRX 600 MHz spectrometer at 298 K in deuterated 50 mM Tris/d₂0, 10 mM MgCl₂, pH 7.8.

**NMR-based Enzyme Assays—** In a typical ¹H NMR experiment, a spectrum of a mixture containing 1 mM Glc-1-P and 1 mM UTP was acquired at t = 0 min. After the addition of 200 ng of purified UGP, ¹H NMR spectra were recorded in 10-min time intervals over 1 h. Suppression of the residual HDO signal was achieved by presaturation with a weak radio frequency field for 1.5 s during the relaxation delay. Data acquisition and processing were performed with XWINNMR software (Bruker) on a Silicon Graphics O2 work station. Fourier transformation and base-line correction were performed for the ¹H NMR experiment at t = 0 min.

**STD-NMR Experiments—** STD-NMR experiments (27) were performed at 298 K and pH 7.8. The protein was saturated on resonance at 0.7 ppm and off resonance at 40 ppm with a cascade of 40 selective Gaussian-shaped pulses of 50-ms duration with a 100-μs delay between each pulse in all STD-NMR experiments. The total duration of the saturation time was set to 2 s. For STD-NMR experiments, 0.6 µM recombinant UGP was used. All investigated ligands were added at a molecular ratio (protein/ligand) of 1:100. A total of 1024 scans/STD-NMR experiment were acquired, and a WATERGATE sequence was used to suppress the residual HDO signal. A lock spin filter with a strength of 5 kHz and a duration of 10 ms was applied to suppress protein background. Relative STD effects were calculated according to the equation:

\[ A_{STD} = \frac{I_{STD} - I_{off}}{I_{off}} \]
a reference spectrum ($I_0$). The STD signal with the highest intensity was set to 100%, and other STD signals were calculated accordingly (28).

RESULTS

Isolation and Functional Characterization of the L. major UDP-glucose Pyrophosphorylase—Because the initial goal of our study was the characterization of UGT from L. major, heterologous complementation in UGT-deficient CHO Lec8 cells was selected as a strategy to clone the gene. Technically, the complementation approach followed a recently established protocol, which allowed the isolation of UGT activities from an Arabidopsis thaliana cDNA library (21). To enable sibling selection, a cDNA library constructed from the promastigote (sandfly) form of L. major in the eukaryotic expression vector pCMV-Script was subdivided into pools of 2/1100 cDNAs. Plasmid cDNA was isolated from individual pools and transiently transfected into Lec8 cells together with a cDNA encoding the rat glucuronyltransferase (16). Complementation of the UGT defect in the presence of the rat glucuronyltransferase results in the cell surface expression of glucuronic acid 1,3-linked to galactose, an epitope recognized by the monoclonal antibody L2-412 (20). After six rounds of sibling selection, a complementing cDNA clone named pACM1 was isolated (data not shown). The isolated cDNA contained an open reading frame of 1485 bp and encoded a protein of 494 amino acids with a calculated molecular mass of 54.5 kDa. To prove that the cloned gene belonged to the nucleotide sugar transporter family, a data base alignment was carried out with the program BLASTX2.2.3 (NCBI). Unexpectedly, this analysis showed that the protein encoded in pACM1 exhibited about 30% identity to eukaryotic UGPs (supplemental Fig. S1). Like the other eukaryotic enzymes, the L. major sequence harbors the characteristic pyrophosphorylase consensus motif (KLNGLGLGTXMGX;K; Fig. S1) known to be essential for catalytic activity (29). Different from all other eukaryotic UGPs, the L. major enzyme contains an insertion of 14 amino acids at position 263 (Fig. S1). L. major UGP Complements the E. coli Mutant DEV6 galU—UGPs catalyze the synthesis of UDP-Glc and PP from Glc-1-P and UTP. UDP-Glc is a central compound in carbohydrate synthesis and in metabolic pathways, since it is required for the entry of galactose into the glycolytic pathway in bacterial and eukaryotic cells (Fig. 1). Consequently, the E. coli galU mutant strain DEV6, which exhibits a defect in the ugp, has lost its ability to ferment galactose. This defect renders the bacterium unable to grow on agar containing galactose as the only carbohydrate source. Upon transformation with the L. major UGP cDNA, the phenotype of the E. coli galU mutant strain DEV6 could be restored, and the mutant was able to grow on MacConkey-galactose agar (data not shown). Taken together, these results unequivocally identified pACM1 to encode a functional UGP. In contrast, the question of why the L. major enzyme was able to complement CHO Lec8 cells remained unanswered.

Lec8 Complementation by L. major but Not Human UGPs—As known from earlier studies, Lec8 cells exhibit a low residual Golgi UDP-Gal transport activity though a functional UGT is missing (19, 30). Therefore, the most likely explanation for the observed phenotypic correction by the L. major UGP was that overexpression elevated the cytoplasmic UDP-Gal concentration and thus forced transport by nucleotide sugar transporters with low affinity for UDP-Gal. To elucidate if a similar effect could be evoked by the overexpression of mammalian UGPs, the cDNAs of the two human UGP-isoforms a and b (23) were isolated and overexpressed in Lec8 cells in parallel to the L. major UGP. Although all recombinant proteins could be clearly identified by Western blotting in lysates of transfected cells, complementation of Lec8 cells

---

**FIGURE 1.** Metabolic activation of galactose depends on UDP-glucose pyrophosphorylase activity. The positioning of UDP-glucose pyrophosphorylase in the pathways leading to UDP-galactose is schematically represented. UDP-glucose, the direct product of the UDP-glucose pyrophosphorylase-catalyzed reaction (bottom), is substrate in the reactions catalyzed by galactose-1-phosphate uridylyltransferase and UDP-galactose 4-epimerase, leading to UDP-galactose. Only the activated sugar, UDP-galactose, can be transported into the Golgi lumen.
was visible only in cells transfected with the L. major enzyme, clearly demonstrating that mammalian and parasite enzymes differ fundamentally in terms of activity. This finding prompted a series of studies designed to obtain insight into regulation, kinetic properties, and substrate recognition of the L. major enzyme.

**L. major UGP Is a Monomer with Simple Michaelis-Menten Kinetics**—UGPs from different eukaryotic cells have been found to form oligomers. Sequestration by oligomerization has recently been suggested to provide an important principle in the regulation of these enzymes. For example, Gomis et al. (31) asked whether the L. major enzyme is regulated in the same manner, we investigated the oligomerization status of recombinantly expressed L. major UGP. A C-terminally His6-tagged form of the protein was expressed in E. coli BL21(DE3), and after purification by Ni2+ chelating chromatography (see Fig. 2A), it was used in size exclusion chromatography to determine the quaternary organization (Fig. 2B). Standard proteins like bovine serum albumin, bovine carbonic anhydrase, and yeast alcohol dehydrogenase were used in calibration runs. The recombinant L. major UGP generated a single peak at a retention volume of 14.2 ml, both in the presence and absence of the substrates UTP and Glc-1-P (Fig. 2B, solid line). In addition, the activity profile (Fig. 2B, dotted line) of all fractions matched the elution profile, showing that the protein was exclusively monomeric. An exactly identical elution profile was obtained using an untagged form of the recombinant protein that was partially purified by anion exchange chromatography (data not shown). This result clearly indicates that the C-terminal tag does not influence the quaternary organization. Moreover, the oligomeric state of the native enzyme was analyzed by loading the cytosolic fraction of L. major cells on the size exclusion chromatography column. Elution was monitored by activity measurements and by Western blotting with a serum specifically recognizing the L. major UGP (Fig. 2C). Both confirmed that the endogenous enzyme migrates indistinguishably from the recombinant forms and, therefore, is a monomer (Fig. 2C).

The determined monomeric state of the L. major UGP was in excellent agreement with the obtained kinetic data. Under in vitro conditions, UGPs are active in the forward (production of UDP-Gl 1c and PP from Glc-1-P and UTP) and reverse reaction (production of UTP and Glc-1-P from PP, and UDP-Glc). Therefore, assay systems measuring both ways were used to kinetically characterize the purified enzyme. The initial enzyme velocities were plotted against substrate concentrations according to Lineweaver-Burk in a double reciprocal manner. The graphs were linear for all substrates, clearly demonstrating that the L. major enzyme obeyed a simple Michaelis-Menten kinetics. This finding was in marked contrast to the biphasic kinetics found for Glc-1-P and UTP of the potato enzyme (32) and to the kinetic anomalies described for the recombinantly expressed human UGPs (23). The calculated $K_m$ values for Glc-1-P (192 $\mu$M), UTP (70 $\mu$M), UDP-Glc (104 $\mu$M), and PP, (200 $\mu$M) were, however, comparable with those obtained for UGPs from mammalian and plant sources (Table 1) (23, 32–36). No significant kinetic differences were obtained for the His6-tagged and the untagged L. major UGP.

**Ligand Binding Analysis by STD-NMR Spectroscopy**—To gain a more detailed picture of the ligand binding properties and substrate selectivity of the L. major UGP, we used STD-NMR spectroscopy (27, 28). This method is based on saturation transfer from the target protein to ligands mediated by spin-diffusion and allows the determination of the binding epitope of the ligand. Ligand protons, which are close to the protein surface, exhibit high STD-NMR signal intensities and are more likely to be involved in substrate binding. Since acquisition of informative data

---

**TABLE 1**

| Reaction | Substrate               | $K_m$ (mM) |
|----------|-------------------------|------------|
| Forward  | UTP                     | 70 ± 5     |
| Reverse  | UDP-glucose             | 192 ± 4    |
|          | Pyrophosphate           | 200 ± 40   |

---

**FIGURE 2.** Purification and quaternary organization of recombinant L. major UDP-glucose pyrophosphorylase. A, purification of E. coli expressed L. major UDP-glucose pyrophosphorylase. The silver-stained SDS-PAGE shown in a documents homogenous purification in a single chromatographic step. Lane 1, the soluble fraction after bacterial lysis; lane 2, the flow-through of the Ni2+ -chelating column; lane 3, the homogenously purified protein. The Western blot shown in b identifies the His6-tagged UDP-glucose pyrophosphorylase using an anti-penta-His antibody, and the blot in c identifies it using a rabbit serum directed against the L. major UGP. B, to determine the quaternary organization, purified UDP-glucose pyrophosphorylase was submitted to size exclusion chromatography. Before sample loading, the column was calibrated with protein standards. Elution positions of standard proteins are indicated by arrows. a, yeast alcohol dehydrogenase, 150 kDa; b, bovine serum albumin, 66 kDa; c, bovine carbonic anhydrase, 29 kDa. The L. major UGP (solid line) elutes at a retention volume of 14.2 ml, corresponding to a protein of 59 kDa. The activity pattern of eluted fractions (dotted line) confirmed migration as a monomer. C, to analyze the oligomerization status of the native protein, the cytosolic fraction of L. major cells was passed over the column. Elution was monitored by activity measurements and by Western blotting using an anti-L. major UGP antisemur. Identical to the recombinant form, the endogenous enzyme is a monomer.

---

**The UDP-glucose Pyrophosphorylase of L. major**

The UGP activity was measured using coupled enzymatic reactions (pH 7.8, 10 mM MgCl2, 25 °C). One substrate was kept at saturating concentration while the concentration of the second substrate was varied. $K_m$ values for the substrates of the forward and reverse reaction were determined in Lineweaver-Burk plots. Simple Michaelis-Menten kinetics were obtained with all substrates.

---

**TABLE 2**

| Reaction | Substrate               | $K_m$ (mM) |
|----------|-------------------------|------------|
| Forward  | UTP                     | 70 ± 5     |
| Reverse  | UDP-glucose             | 192 ± 4    |
|          | Pyrophosphate           | 200 ± 40   |

---

**FIGURE 2.** Purification and quaternary organization of recombinant L. major UDP-glucose pyrophosphorylase. A, purification of E. coli expressed L. major UDP-glucose pyrophosphorylase. The silver-stained SDS-PAGE shown in a documents homogenous purification in a single chromatographic step. Lane 1, the soluble fraction after bacterial lysis; lane 2, the flow-through of the Ni2+ -chelating column; lane 3, the homogenously purified protein. The Western blot shown in b identifies the His6-tagged UDP-glucose pyrophosphorylase using an anti-penta-His antibody, and the blot in c identifies it using a rabbit serum directed against the L. major UGP. B, to determine the quaternary organization, purified UDP-glucose pyrophosphorylase was submitted to size exclusion chromatography. Before sample loading, the column was calibrated with protein standards. Elution positions of standard proteins are indicated by arrows. a, yeast alcohol dehydrogenase, 150 kDa; b, bovine serum albumin, 66 kDa; c, bovine carbonic anhydrase, 29 kDa. The L. major UGP (solid line) elutes at a retention volume of 14.2 ml, corresponding to a protein of 59 kDa. The activity pattern of eluted fractions (dotted line) confirmed migration as a monomer. C, to analyze the oligomerization status of the native protein, the cytosolic fraction of L. major cells was passed over the column. Elution was monitored by activity measurements and by Western blotting using an anti-L. major UGP antisemur. Identical to the recombinant form, the endogenous enzyme is a monomer.
FIGURE 3. Ligand binding measured by STD-NMR. Spectra are shown for UDP-glucose (A), UTP (B), UDP (C), and UDP-galactose (D). $^1$H NMR reference spectra used to correctly assign STD signals are given at the bottom of each panel. Relative STD-NMR signals were calculated and are indicated for each proton. UTP and UDP-glucose show strong STD-NMR signals, whereas UDP and UDP-galactose do not bind to the enzyme.
essentially depends on the integrity of the reaction, STD-NMR studies were preceded by a real time recording of the enzyme catalysis using $^1$H NMR. Under the applied assay conditions, the enzyme was active in the forward (synthesis of UDP-Glc) and the reverse (formation of UTP and Glc-1-P) reaction, and both reactions reached equilibrium (data not shown).

Because kinetic studies carried out with the *L. major* (this study) and other eukaryotic UGPs (33, 37) had shown that UDP-Glc and UTP bind with high affinity, whereas binding of Glc-1-P required preloading of the enzyme with UTP, STD-NMR-based ligand binding studies were first recorded for UDP-Glc (Fig. 3A) and UTP (Fig. 3B). It is immediately obvious that both nucleotide sugar (UDP-Glc) and nucleotide (UTP), make major contacts to the enzyme via the ribose. Thereby, the H1 proton of the ribose (H1 (Rib)) generates the strongest STD-NMR signal. Whereas the signal intensities obtained for other protons are in about the same regime in UTP and UDP-Glc, intensified contacts are made by the H5 proton of the nucleotide base (H5 (Uri)) and the H4 proton of the ribose (H4 (Rib)) in the product UDP-Glc.

All of the sugar ring protons generated STD-NMR signals; however, the strongest contact was obtained for the proton in position C4, which is the substrate-defining position of the sugar ring. The pivotal role of this position in binding to the active site was further highlighted by the virtual absence of binding capability of the activated epimer UDP-Gal (Fig. 3D). The lack of saturation transfer in STD-NMR correlates perfectly with the extremely low UDP-Gal production rate measured in the activity assay. Furthermore, the STD-NMR experiments clearly demonstrate that nucleotide binding essentially depends on the presence of either the glucose moiety or the γ-phosphate. Fragments like UDP (see Fig. 3C) and UMP (data not shown) did not bind to the enzyme. To assign the correct proton position to the corresponding STD-NMR signals, a reference $^1$H NMR spectrum of each substrate was determined (Fig. 3, A–D, bottom panels).

Sequential Substrate Binding Mode of *L. major* UGP—Based on product inhibition assays, eukaryotic UGPs were suggested to follow a sequential binding mode with UTP preceding binding of Glc-1-P (33, 37). Here we applied STD-NMR methods to directly illustrate the postulated mechanism. As described in the previous experiments, $^1$H NMR spectra were recorded to assign signals and to ensure that the reaction takes place under the applied assay conditions (Fig. 4; a, Glc-1-P; b, UDP-Glc; c, reaction mixture containing UTP plus Glc-1-P). To test if Glc-1-P per se is able to bind to UDPG, a mixture of Glc-1-P and enzyme was analyzed. The complete absence of Glc-1-P signals in the STD-NMR spectrum (Fig. 4d) clearly demonstrated that Glc-1-P was unable to make specific contacts to the enzyme. However, after the addition of UTP, specific STD-NMR signals were recorded for both the H2 and H4 protons of Glc-1-P (Fig. 4e). For the first time, these results clearly demonstrate direct proof for the sequential mode of substrate binding in a UGP. Binding of UTP seems to be required to induce the opening or formation of the substrate binding pocket for Glc-1-P.

Based on this observation, we were then interested in investigating if the presumed change in conformation that is necessary to allow entry of Glc-1-P could be induced by other UGP ligands like PP, and UDP-Glc or the nucleotides UDP and UMP. However, none of these compounds were able to mediate Glc-1-P binding to the enzyme. Even after the simultaneous addition of PP, and UMP, used to mimic the trisphosphate group of UTP, no Glc-1-P signals were obtained (data not shown). These results strongly support the notion that the γ-phosphate group in UTP is mandatory to induce the conformational change required for the binding of Glc-1-P.

**FIGURE 4.** UDP-glucose pyrophosphorylase binds substrates in an ordered bi-bi mechanism. STD-NMR has been used to investigate the mechanism of substrate binding to UDP-glucose pyrophosphorylase. The spectra a and b represent $^1$H NMR reference measurements of glucose-1-phosphate and UDP-glucose. The $^1$H NMR spectrum c confirms that formation of UDP-glucose from UTP and glucose 1-phosphate takes place in the NMR tube. Glucose 1-phosphate, if added as the only substrate, does not bind to the enzyme, as indicated by the complete absence of STD-NMR signals in spectrum d. However, after supplementation of UTP, STD-NMR signals became visible for both educts glucose-1-phosphate and UTP as well as for the product UDP-glucose (e). These results clearly demonstrate that binding of UTP is a prerequisite for the entry of the second substrate glucose-1-phosphate.

**DISCUSSION**

In this study, a functionally active UGP from *L. major* has been cloned and biochemically characterized. Unexpectedly, the gene was isolated in a heterologous complementation cloning approach with an *L. major* cdNA library and CHO Lec8 cells as hosts. Because UGP is of central importance in both anabolic and catabolic pathways of the *Leishmania* parasite and thus may represent an attractive drug target, we decided to study the biochemical and functional features of this enzyme in detail.

Whereas the *L. major* UGP described in this study provides the first molecular information on a protozoan enzyme, UGPs have been cloned from numerous eukaryotic and prokaryotic origins. An in-depth homology analysis carried out recently to reveal evolutionary relatedness showed that all eukaryotic UGPs isolated from plant, animal, yeast, and slime mold form separate but tightly linked clades in a parsimony tree (38). In contrast, the prokaryotic UGPs form a distinct clade showing no significant sequence homology to eukaryotic UGPs. The newly identified *L. major* enzyme grouped into the clade of eukaryotic UGPs but belonged to a separate branch, including putative UGPs from the protozoan parasites*Trypanosoma brucei* and *Leishmania infantum* (Fig. 5).

The central position of the UGP in eukaryotic cells is illustrated in Fig. 1. Formation of UDP-Glc by UGP is a prerequisite for the activation of galactose either by nucleotide transfer onto Gal-1-P or by epimerization of UDP-Glc to UDP-Gal. Therefore, it is most likely that the observed complementation of Lec8 cells after expression of the *L. major* UGP represented the result of elevated UDP-Gal pools and subsequent activation of Golgi transport systems with low affinity for UDP-Gal. In fact, it is known from earlier studies that Lec8 cells express low amounts of galactosylated proteins and lipids (about 15% compared with wild type cells) and that this surface expression of galacto-glycoconjugates corre-
lates with a residual (about 5% of wild type) UDP-Gal transport activity into the Golgi (30). Because a deletion of 99 nucleotides completely abolishes the generic UDP-Gal transport in Lec8 cells (19), the viable transport in Lec8 must be mediated by another nucleotide sugar transporter, potentially a multisubstrate nucleotide sugar transporter as identified in Caenorhabditis elegans (39). It is important to mention in this context that in mammals, alternative transport activities exist for UDP-GlcNAc (40) and most probably for GDP-fucose (41). Our attempts to additionally clone a UDP-Gal transport activity from the L. major cDNA library failed due to the broad abundance of the UGP-encoding cDNA (64 of 86 cDNA library pools contained a functional UGP) and its strong complementary activity in CHO Lec8 cells. The prevalence of UDP in the cDNA library is in line with the fact that the promastigote (sandfly) form of the parasite up-regulates the expression of galactose-containing glycoconjugates (reviewed in Ref. 2) and again supports the role played by UGP in controlling the flux through these pathways.

UGPs isolated from L. major, but not the enzymes isolated from humans (23), were able to complement Lec8 cells. Based on this observation, we postulated that the L. major enzyme may lack regulation in the heterologous system. A mechanism suggested for the regulation of UGPs is sequestration of the active monomer by oligomerization (31, 42). This regulatory mechanism received confirmation by the resolution of the crystal structure of the closely related human UDP-GlcNAc pyrophosphorylase (AGX (29)). The AGX protein, which crystallized as a dimer in the presence of its products UDP-GlcNAc or UDP-GalNAc, was used as a template for three-dimensional homology models of the L. major cDNA. The monomeric form remains an unanswered question. Human AGX exists in two splice variants, AGX1 and AGX2, which vary in the length of the COOH-terminal domain (AGX2). In L. major, the AGX1 and AGX2 genes were aligned using ClustalW (program Megalign, Lasergene 6).

In this study, STD-NMR spectroscopy has been used to resolve binding of ligands to L. major UGP. ’H NMR studies were carried out under controlled physiological conditions to ensure good enzymatic activity and to ascertain unequivocal identification of sugar nucleotide and nucleoside phosphate. As in other nucleotide sugar binding enzymes (e.g. UDP-GlcNAc 2-epimerase/N-acetamannosamine kinase) (43–45), the nucleotide moiety receives the largest amount of saturation transfer. In particular, H1 of the ribose and H5 of the uridine ring are in intimate contact with the UGP protein. However, in contrast to UDP-GlcNAc 2-epimerase/N-acetamannosamine kinase, L. major UGP did not exhibit binding properties for UDP and UDP. The third phosphate in UTP is essential for binding to the L. major UGP and can only be replaced by the hexopyranose ring of glucose. Interestingly, the relative STD-NMR signal intensities of H5 and H6 protons of the uracil moiety and the H4 proton of the ribose moiety in the product UDP-Glc are noticeably increased compared with the STD-NMR spectrum of the educt UTP (Fig. 3, compare A and B) and suggest that the formation of the glycosidic linkage is likely to be accompanied by a conformational change within the protein active site.

Despite the obvious importance of the nucleotide part in binding, the sugar moiety has a great impact on substrate specificity in L. major UGP. This fact is clearly visible by the lack of saturation transfer to UDP-Gal in Fig. 3D. Epimerization of position 4 of the hexose is sufficient to prevent binding. This pronounced substrate specificity mediated by the correct orientation of the C4 hydroxyl group marks a clear difference to AGX, where the crystal structure has demonstrated that binding of UDP-GlcNAc and of the C4-epimer UDP-GalNAc proceed without significant structural rearrangements.

For the first time, we could show, by direct measurement, that UGP has no affinity to the substrate Glc-1-P if UTP is absent. With the experiment shown in Fig. 4, we confirm earlier product inhibition studies carried out with UGPs from various sources that support an ordered
The UDP-glucose Pyrophosphorylase of L. major

The bi-bi reaction mechanism for UGPs (33, 37). The complete absence of a Glc-1-P signal in the STD-NMR experiment and the saturation transfer to protons of the glucose moiety after UTP addition allow us to conclude that binding of the cofactor UTP induces a conformational change from “closed” to “open,” enabling entry of Glc-1-P. Interestingly, the complex formed between UGP and UDP-Glc did not facilitate binding of Glc-1-P. This phenomenon (Glc-1-P does not compete with the complex formed between UGP and UDP-Glc did not facilitate binding of Glc-1-P) is in agreement with the ordered bi-bi mechanisms, where competition occurs between the first substrate to add (UTP) and the last product to be released from the enzyme (UDP-Glc) (46).

In summary, this study provides the first molecular characterization of a protozoan UGP and highlights catalytic features that differentiate the parasite enzyme from its mammalian counterparts. Using STD-NMR, the binding of ligands, including the natural substrates UDP-Glc for the binding to the active site pocket) is in agreement with the parasite enzyme.

Acknowledgments—We are thankful to Drs. M. A. J. Ferguson and T. K. Smith (University of Dundee) for L. major lysates and to our colleagues Drs. K. Stummeyer, A.-K. Münnst-Kühnel, M. Mühlenhoff, H. Bakker, F. Routier, and M. Oschlies for helpful discussions and critical reading of the manuscript. We thank Jana Fähring for excellent technical assistance.

REFERENCES

1. WHO Expert Committee (1984) The Leishmaniasis, World Health Organization, Geneva
2. Naderer, T., Vince, J. E., and McConville, M. J. (2004) Curr. Mol. Med. 4, 649–665
3. Sacks, D. L., Modi, G., Rowton, E., Spalth, G., Epstein, L., Turco, S. J., and Beverley, S. M. (2003) Proc. Natl. Acad. Sci. U. S. A. 97, 406–411
4. Reiter, W. D., and Vanzyn, G. F. (2001) Plant Mol. Biol. 47, 95–113
5. Holden, H. M., Rayment, I., and Thoden, J. B. (2003) J. Biol. Chem. 278, 43885–43888
6. Zhang, K., Barron, T., Turco, S. J., and Beverley, S. M. (2004) Mol. Biochem. Parasitol. 136, 11–23
7. Spath, G. F., Garraway, L. A., Turco, S. J., and Beverley, S. M. (2003) Proc. Natl. Acad. Sci. U. S. A. 100, 9456–9451
8. McConville, M. J., and Ferguson, M. A. (1993) Biochem. J. 294, 305–324
9. Turco, S. J., Orlandi, P. A., Jr., Homans, S. W., Ferguson, M. A., Dwek, R. A., and Rademacher, T. W. (1989) J. Biol. Chem. 264, 6711–6715
10. McConville, M. J., Orlandi, P. A., Jr., Homans, S. W., Thomas-Oates, J. E., Dell, A., and Bacic, A. (1990) J. Biol. Chem. 265, 7385–7394
11. Pedersen, L. L., and Turco, S. J. (2003) Cell Mol. Life Sci. 60, 259–266
12. Pan, F., Jackson, M., Ma, Y., and McNeil, M. (2001) J. Bacteriol. 183, 3991–3998
13. Bakker, H., Kleczka, B., Gerardy-Schahn, R., and Routier, F. H. (2005) Biochem. J. 386, 657–661
14. Beverley, S. M., Owens, K. L., Showalter, M., Griffith, C. L., Doering, T. L., Jones, V. C., and McNeil, M. R. (2005) Eukaryot. Cell 4, 1147–1154
15. McConville, M. J., Mullin, K. A., Ilgoutz, S. C., and Teasdale, R. D. (2002) Microbiol. Mol. Biol. Rev. 66, 122–154
16. Terayama, K., Saka, S., Seiki, T., Miki, Y., Nakamura, A., Kozutsu, Y., Takio, K., and Kawasaki, T. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 6093–6098
17. Remes, B., and Elsevier, D. (1980) J. Bacteriol. 143, 1054–1056
18. Munster, A. K., Ekhart, M., Potvin, B., Mühlenhoff, M., Stanley, P., and Gerardy-Schahn, R. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 9140–9145
19. Oelmann, S., Stanley, P., and Gerardy-Schahn, R. (2001) J. Biol. Chem. 276, 26291–26300
20. Kruse, J., Mühlenhoff, M., Wernicke, H., Faisner, A., Sommer, I., Goridis, C., and Schachner, M. (1984) Nature 311, 153–155
21. Bakker, H., Routier, F., Oelmann, S., Jordi, W., Lommen, A., Gerardy-Schahn, R., and Bosch, D. (2005) Glycobiology 15, 193–201
22. Peng, H. L., and Chang, H. Y. (1993) FEBS Lett. 329, 153–158
23. Duggleby, R. G., Chao, Y. C., Huang, J. G., Peng, H. L., and Chang, H. Y. (1996) Eur. J. Biochem. 235, 173–179
24. Laemmli, U. K. (1970) Nature 227, 680–685
25. Heukeshoven, J., and Dernick, R. (1988) Electrophoresis 9, 28–32
26. Corpet, F. (1988) Nucleic Acids Res. 16, 10881–10890
27. Mayer, M., and Meyer, B. (1999) Angew. Chem. Int. Ed. Engl. 38, 1784–1788
28. Mayer, M., and Meyer, B. (2001) J. Am. Chem. Soc. 123, 6108–6117
29. Peneff, C., Ferrer, P., Charrier, V., Taburet, Y., Monnier, C., Zambroni, V., Winter, J., Hartho, M., Fassy, F., and Bourne, Y. (2001) EMBO J. 20, 6191–6202
30. Deutscher, S. L., and Hirschberg, C. B. (1986) J. Biol. Chem. 261, 96–100
31. Martz, F., Wilczynska, M., and Kleczkowski, L. A. (2002) Biochem. J. 376, 295–300
32. Sowokinos, J. R., Spychalla, J. P., and Desborough, S. L. (1993) Plant Physiol. 101, 1073–1080
33. Nakano, K., Omura, Y., Tagaya, M., and Fukui, T. (1989) J. Biochem. (Tokyo) 106, 528–532
34. Turnquist, R. L., Gillett, T. A., and Hansen, R. G. (1974) J. Biol. Chem. 249, 7695–7700
35. Tsuboi, K. K., Fukunaga, K., and Petricciani, J. C. (1969) J. Biol. Chem. 244, 1008–1015
36. Knop, J. K., and Hansen, R. G. (1970) J. Biol. Chem. 245, 2499–2504
37. Elling, L., and Kula, M. R. (1994) Biotechnol. 34, 157–163
38. Geider, M., Wilczynska, M., Karpinski, S., and Kleczkowski, L. A. (2004) Plant Mol. Biol. 56, 783–794
39. Berninsone, P., Hwang, H. Y., Zemtseva, I., Horvitz, H. R., and Hirschberg, C. B. (2001) Proc. Natl. Acad. Sci. U. S. A. 98, 3738–3743
40. Ashikov, A., Routier, F., Fuhlrott, J., Helmus, Y., Wild, M., Gerardy-Schahn, R., and Bakker, H. (2005) J. Biol. Chem. 280, 27230–27235
41. Hong, K., Ma, D., Beverley, S. M., and Turco, S. J. (2000) Biochemistry 39, 2013–2022
42. Kleczkowski, L. A., Martz, F., and Wilczynska, M. (2005) Phytochemistry 66, 2815–2821
43. Benie, A. J., Blume, A., Schmidt, R. R., Reutter, W., Hinderlich, S., and Peters, T. (2004) J. Biol. Chem. 279, 55722–55727
44. Blume, A., Benie, A. J., Stolz, F., Schmidt, R. R., Reutter, W., Hinderlich, S., and Peters, T. (2004) J. Biol. Chem. 279, 55715–55721
45. Yuan, Y., Wen, X., Sanders, D. A., and Pinto, B. M. (2005) Biochemistry 44, 14080–14089
46. Voet, D., Voet, J. G., and Pratt, C. W. (2002) Fundamentals of Biochemistry, pp. 331–410, John Wiley & Sons, Inc., New York