

## Hemoglobin Receptor in *Leishmania* Is a Hexokinase Located in the Flagellar Pocket\*

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**Hb endocytosis in *Leishmania* is mediated through a 46-kDa protein located in the flagellar pocket. To understand the nature of the Hb receptor (HbR), we have purified the 46-kDa protein to homogeneity from *Leishmania* promastigote membrane. Purified HbR specifically binds Hb. The gene for HbR was cloned, and sequence analysis of the full-length HbR gene indicates the presence of hexokinase (HK) signature sequences, ATP-binding domain, and PTS-II motif. Four lines of evidence indicate that HbR in *Leishmania* is a hexokinase: 1) the recombinant HbR binds Hb, and the Hb-binding domain resides in the N terminus of the protein; 2) recombinant proteins and cell lysate prepared from HbR-overexpressing *Leishmania* promastigotes show enhanced HK activity in comparison with untransfected cells; 3) immunolocalization studies using antibodies against the N-terminal fragment (Ld-HbR- $\Delta$ C) of Ld-HbR indicate that this protein is located in the flagellar pocket of *Leishmania*; and 4) binding and uptake of <sup>125</sup>I-Hb by *Leishmania* is significantly inhibited by anti-Ld-HbR- $\Delta$ C antibody and Ld-HbR- $\Delta$ C, respectively. Taken together, these results indicate that HK present in the flagellar pocket of *Leishmania* is involved in Hb endocytosis.**

*Leishmania donovani*, a protozoan parasite, is the etiological agent of the fatal form of human visceral leishmaniasis or kala-azar (1), affecting millions of people worldwide. In common with most trypanosomatids, *Leishmania* lack a complete heme biosynthetic pathway (2) and must acquire heme from the external sources to sustain their growth (3). The majority of the heme in the host is sequestered in erythrocytes in the form of hemoglobin. Consequently, Hb-binding proteins have been identified in some pathogens, e.g. *Neisseria gonorrhoeae*, *Haemophilus influenzae*, *Porphyromonas gingivalis*, and so forth (1, 4, 5). However, information is scanty regarding the role of these proteins in the physiology of these pathogens. Because such organisms often lack the ability for *de novo* biosynthesis of heme (6, 7), it appears likely that these proteins may be involved in the internalization and subsequent intracellular deg-

radation of Hb to generate heme, which is essential for their growth. Recently, we have shown that Hb endocytosis in *Leishmania* is mediated through a 46-kDa protein located in the flagellar pocket of *Leishmania* (8), and signals mediated through the cytoplasmic C-terminal tail of this protein are essential for targeting the protein-bound Hb to the late compartment (9), probably to generate intracellular heme. Moreover, a few receptor systems have been identified on trypanosomatid parasites such as *Trypanosoma* and *Leishmania* that mediate the uptake of low density lipoprotein and transferrin, presumably for efficient supply of nutrients such as cholesterol and metal ions such as iron required for rapid growth (10–12). However, the mechanism of endocytosis and its regulation are not very well characterized in parasitic protozoa (13).

In the present investigation, we describe the purification and characterization of a 46-kDa HbR<sup>1</sup> from the *L. donovani* promastigotes membrane to homogeneity. The sequence of the cloned HbR gene and its biochemical characterization reveal that HbR is a HK located in the flagellar pocket of *Leishmania* and is involved in Hb endocytosis.

### EXPERIMENTAL PROCEDURES

**Reagents**—*N*-Hydroxysuccinimido-biotin and avidin-horseradish peroxidase (HRP) were purchased from Vector Laboratories (Burlingame, CA). All HRP-labeled secondary antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Texas Red-labeled goat anti-mouse IgG was purchased from Jackson Immunoresearch Laboratory (West Grove, PA). Alexa Fluor-labeled goat anti-mouse IgG, SYTO dyes, and Prolong antifade kit were obtained from Molecular Probes (Eugene, OR). pXG vector used for overexpression of HbR in *Leishmania* was received as a gift from Dr. S. M. Beverley (Washington University, St Louis, MO). ECL reagents and SP-Sepharose fast flow matrix were obtained from Amersham Biosciences. All other reagents used were of analytical grade. Unless otherwise stated, all reagents were obtained from Sigma. Antibodies against the purified HbR and recombinant N-terminal fragment of Ld-HbR were raised in mice by a standard technique (14). Hb was biotinylated using *N*-hydroxysuccinimido-biotin as described previously (15).

***Leishmania***—Promastigotes of *L. donovani* were obtained from the Indian Institute of Chemical Biology (Kolkata, India). The cells were routinely maintained on blood agar slants containing brain heart infusion, glucose, peptone, sodium chloride, blood, and gentamycin (16). For experimental purposes, cells were harvested from a 3-day-old culture using phosphate (10 mM; pH 7.2)-buffered saline (0.15 M).

**Purification of HbR from *Leishmania* Promastigotes**—Membrane fraction from *L. donovani* promastigotes was prepared as described previously (8, 17). Briefly, cells were incubated for 1 h at 4 °C in hypotonic Tris-HCl buffer (5 mM; pH 7.2) and subsequently lysed by sonication (3 × 20 s). The unbroken cells and other cell debris were separated by low-speed centrifugation (500 × *g* for 10 min). The result-

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AY659996.

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<sup>1</sup> The abbreviations used are: HbR, hemoglobin receptor; HK, hexokinase; HRP, horseradish peroxidase; PBS, phosphate-buffered saline; LSB, low salt buffer; BHb, biotinylated hemoglobin; GST, glutathione *S*-transferase; RACE, rapid amplification of cDNA ends.

ant supernatant was again centrifuged at  $100,000 \times g$  for 1 h at 4 °C to obtain membrane protein-enriched fraction. Finally, membrane proteins were extracted with PBS containing 2%  $\beta$ -octyl glucoside at 4 °C overnight and separated from residual debris by centrifugation at  $100,000 \times g$  for 1 h at 4 °C.

In order to enrich the 46-kDa HbR, membrane proteins (16 mg) were sequentially precipitated with 27% and 50% ammonium sulfate, and the precipitate obtained after 50% ammonium sulfate treatment was dissolved and dialyzed against a low salt buffer (LSB; 50 mM Tris-HCl, 1 mM EDTA, 1 mM dithiothreitol, 1% Triton X-100, and 50 mM NaCl, pH 7.5, containing protease inhibitor). Subsequently, dialysate containing membrane proteins (8 mg) was loaded onto a pre-equilibrated (LSB with 2 M urea) SP-Sepharose fast flow column and allowed to bind to the matrix. Unbound proteins were removed from the matrix by washing the column with 20 volumes of LSB. Finally, bound proteins were eluted with a linear gradient of salt (50–700 mM NaCl in LSB), and different fractions were collected. The protein profile of each fraction was determined by SDS-PAGE followed by silver staining. HbR-containing fractions were pooled and dialyzed against LSB.

**Detection of Hb Binding with HbR**—Determination of Hb binding with HbR was carried out using biotinylated hemoglobin (Bhb) by a modified enzyme-linked immunosorbent assay as described previously (18). Briefly, 500 ng of the native or recombinant HbR (10  $\mu$ g/ml) was incubated in an enzyme-linked immunosorbent assay plate for 1 h at 37 °C in 50  $\mu$ l of coating buffer (0.1 N sodium carbonate buffer, pH 9) to coat the plastic wells with the protein, and subsequently, wells were washed three times with PBST (PBS containing 0.1% Tween 20) and incubated for 1 h at 37 °C in blocking buffer (PBST containing 2% gelatin). Wells were washed three times with PBST and incubated with different concentrations of Bhb in 100  $\mu$ l of PBS for 1 h at 37 °C to allow binding. To determine the binding of Bhb, wells were washed and incubated with 100  $\mu$ l (1  $\mu$ g/ml) of avidin-HRP for 30 min at 37 °C. Finally, unbound avidin-HRP was removed by washing with PBST, and the HRP activity present in each well was measured by a standard procedure (19) and expressed as relative binding of Hb with test protein. Similarly, binding of Bhb with immobilized HbR was also determined in the presence of unlabeled Hb (10  $\mu$ g). To determine the specificity, binding of Bhb (25 ng) with immobilized HbR (500 ng) was carried out in the presence of 100-fold excess of different heme- or iron-containing proteins (2.5  $\mu$ g) under similar conditions.

**Detergent Solubilization and Phase Separation of HbR**—To determine whether the HbR in *Leishmania* is an integral membrane protein, membrane fraction was prepared (17) and subjected to analytical phase separation in nonionic detergent Triton X-114 as described previously (20). Subsequent to phase separation by Triton X-114, the distribution of HbR was determined by Western blot analysis using anti-HbR antibody.

**Cloning and Expression of HbR from *L. donovani***—The purified protein was subjected to tryptic digestion, and the peptides obtained were purified and sequenced by mass spectrometry in a commercial facility (W. M. Keck Biomedical Mass Spectrometry Laboratory, University of Virginia). Subsequently, BLAST analysis of peptides showed that peptides 17 and 14 have significant homology with the *Leishmania major* partial HK sequence reported in the GenBank™ data base with accession numbers AF202062 and AQ848098, respectively, and these peptides are located in the middle of the reported full-length HK sequence of *Trypanosoma brucei* (21). Accordingly, forward and reverse primers were designed against peptide 17 (5'-GTGGATCCAGTGCCTTGGTTGGCGATGC-3') and peptide 14 (5'-GTGAATTCCTTGGAGTCGAAGTTACCGCA-3') and used to amplify an internal region of HbR by reverse transcription-PCR using *L. donovani* cDNA. Briefly, total RNA isolated from *Leishmania* promastigotes using Trizol reagent (Invitrogen) was used for cDNA synthesis using Super ScriptII reverse transcriptase (Invitrogen) according to the manufacturer's instructions. Subsequently, PCR was performed using the above-mentioned primers and Platinum HiFidelity Taq polymerase (Invitrogen) in a PerkinElmer Life Sciences thermocycler for 30 cycles (denaturation at 94 °C for 30 s, annealing at 62 °C for 30 s, and extension at 68 °C for 2 min). The 5' end of the gene was amplified using a forward primer against the mini-exon (22) sequence (5'-CTCGGAATTCACGCTATATAAGTATCAGTTTC-TGTACTTTATTG-3') and a reverse primer against peptide 17 (5'-GTGAATTCATCGCCAACCAAGGGCACT-3') by PCR under similar conditions. Similarly, the 3' end of the HbR gene was amplified using a 3'-RACE kit (Invitrogen) according to the manufacturer's instructions, with a forward primer designed against peptide 14 (5'-GTGGATCCGAGTGGCGTAACTTCGACTCC-3') under identical conditions. The PCR products were cloned into pGEMT-easy vector (Promega) and sequenced using m13 universal primers in an automated sequencer. From

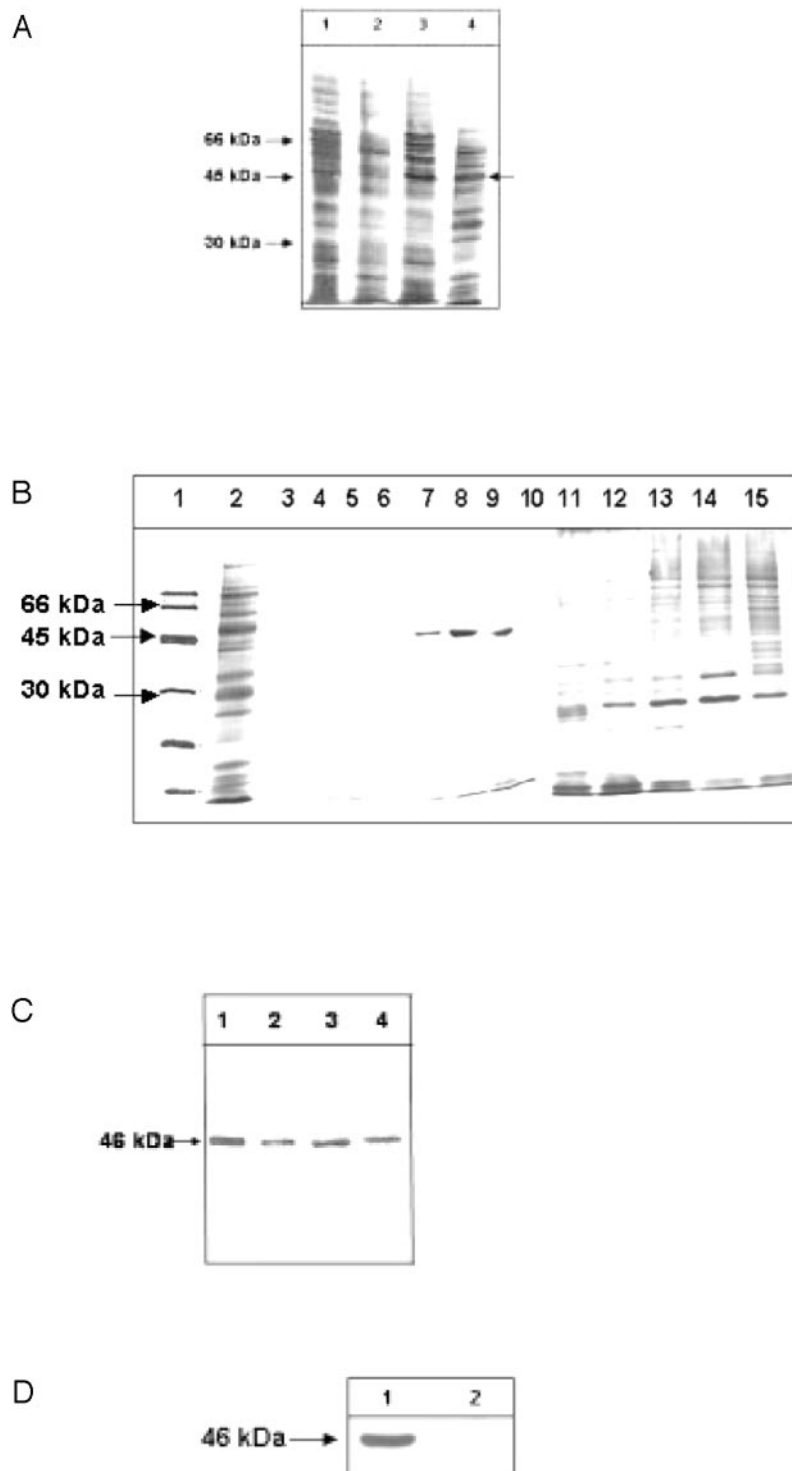
the sequence of the 5'- and 3'-end fragments, the start and stop codons of the gene were identified. Subsequently, forward (5'-GTGGATCCATGGCCACCCGCGTGAAC-3') and reverse (5'-GTGAATTCCTACTTCT-TGTTGACGGCCA-3') primers designed against the start and stop codons, respectively, were used to amplify the open reading frame of the putative HbR from *L. donovani* cDNA by PCR under similar conditions. The full-length gene was cloned into pGEMT-easy vector (Promega), sequenced, subsequently subcloned into pGEX-4T-2 vector (Amersham Biosciences), and transformed into *Escherichia coli*. Transformed *E. coli* were grown in LB and induced with isopropyl  $\beta$ -D-thiogalactopyranoside, and recombinant GST-HbR was purified by a standard procedure using reduced glutathione beads. Similarly, the full-length gene was also subcloned into pET 28a and pET 28b vectors (Novagen) and transformed into *E. coli* to produce recombinant protein with a His<sub>6</sub> tag in either the N (His-HbR) or C terminus (HbR-His) of the HbR, respectively, according to manufacturer's protocol.

**Overexpression of HbR in *Leishmania* Promastigotes**—To overexpress HbR in *Leishmania* promastigotes, pXG vector (23) was linearized by digestion with BamHI and subsequently partially digested with EcoRI, followed by ligation with BamHI-EcoRI-digested full-length HbR. *Leishmania* promastigotes were transfected with this construct using a standard protocol (24). Briefly, *Leishmania* cells were grown to the late log phase ( $\sim 1.0 \times 10^7$  cells/ml) at 23 °C in M199 medium supplemented with 10% fetal calf serum. Cells were resuspended at a density of  $1.0 \times 10^8$  cells/ml in HEPES-buffered saline (21 mM HEPES, 137 mM NaCl, 5 mM KCl, 0.7 mM NaH<sub>2</sub>PO<sub>4</sub>, and 6 mM glucose, pH 7.4). Cells (0.4 ml) were transferred to a pre-cooled electroporation cuvette, and an appropriate construct of chilled DNA (40  $\mu$ g) was added, followed by electroporation using a GenePulser (Bio-Rad) to facilitate DNA uptake by cells. Cells were then incubated on ice for 10 min and transferred into drug-free medium for 30 h at 23 °C. Subsequently, stable clones were selected in the presence of G418 antibiotic (30  $\mu$ g/ml). Overexpression of HbR was confirmed by Western blotting using specific antibody.

**Determination of Activity of HbR**—HK activity of HbR was measured either from the respective recombinant HbR proteins (10  $\mu$ g) or from the cell lysate (10  $\mu$ g) prepared from the HbR-overexpressing *Leishmania* promastigotes using a coupled reaction as described previously (25), with minor modifications. *Leishmania* promastigotes overexpressing HbR were lysed by PBS containing 0.2% Triton X-100 and protease inhibitors for 30 min on ice and used in the HK assay. Briefly, HK activity was determined using standard reaction mixtures (0.6 ml) containing 50 mM Tris (pH 8.0), 5 mM MgCl<sub>2</sub>, 4 mM glucose, 5.3 mM ATP, 0.72 mM NADP, and 2 units/ml *Leuconostoc mesenteroides* glucose-6-phosphate dehydrogenase (type XXIV from *L. mesenteroides*; Sigma). The reaction was initiated by the addition of 10  $\mu$ g of cell extract or recombinant HbR, and the initial velocity of NADP reduction ( $\epsilon_{\text{NADPH}} = 6.22 \times 10^6 \text{ cm}^{-2}$ ) was measured at 30 °C as the change in absorbance at 340 nm at 30-s intervals. Protein concentration of the cell lysate was determined by the bicinchoninic acid method (26).

**Determination of Binding and Uptake of <sup>125</sup>I-Hb by *Leishmania***—Hemoglobin binding and uptake by *Leishmania* were determined as described previously (8). Briefly, Hb was labeled with Na<sup>125</sup>I by iodine monochloride-catalyzed reaction, and >99% of the radioactivity was acid-precipitable with a specific activity of 165 cpm/ng Hb. To determine the inhibition of <sup>125</sup>I-Hb binding with cells by indicated antibodies, cells ( $1 \times 10^7$ ) were washed and pre-incubated with serum containing the respective antibody (1:50) in RPMI 1640 in the presence of 1 mg/ml bovine serum albumin for 10 min at 4 °C, and then cells were incubated at 4 °C with 6  $\mu$ g/ml <sup>125</sup>I-Hb. After 2 h, cells were washed to remove the unbound radioactivity. The cell pellet was finally dissolved in 0.1 N NaOH, and an aliquot was used to determine the cell-associated radioactivity. Similarly, cells were incubated at 23 °C with 1  $\mu$ g/ml <sup>125</sup>I-Hb for 4 h in the presence or absence of the same concentration of different fragments of the HbR to determine the uptake of Hb. Results were expressed as ng Hb bound/mg cell protein.

**Immunolocalization of HbR and Hb Binding in *Leishmania***—In order to localize Ld-HbR in *Leishmania* promastigotes, cells were washed three times with PBS and incubated with anti-Ld-HbR- $\Delta$ C antibody (1:50) for 1 h at 4 °C. Subsequently, cells were washed three times with PBS and probed with Texas Red-conjugated goat anti-mouse secondary antibody for 1 h at 4 °C to detect the primary antibody binding sites. Promastigotes were pre-incubated with SYTO-14 for 30 min at 23 °C in PBS to label the nucleus. Slides were mounted with antifade reagents and viewed in a LSM 510 confocal microscope using an oil immersion objective.



**FIG. 1. Purification of HbR from *L. donovani* promastigotes.** A, the detergent-extracted membrane proteins were sequentially precipitated with 27% and 50% ammonium sulfate to enrich 46-kDa HbR and analyzed by 12% SDS-PAGE as described under "Experimental Procedures." Membrane proteins, lane 1; 27% ammonium sulfate precipitate, lane 2; 27% ammonium sulfate supernatant, lane 3; 50% ammonium sulfate precipitate, lane 4. B, separation of 46-kDa HbR from other membrane proteins by ion-exchange chromatography as described under "Experimental Procedures." Fractions were collected, and aliquots were analyzed by SDS-PAGE followed by silver stain (lanes 3–15). Molecular mass markers (lane 1) and dialysate loaded onto the column (lane 2) are shown in the respective lanes. C, determination of Hb binding activity of the purified 46-kDa protein. Proteins from fractions 5–7 (lanes 7–9 of B) were transferred onto nitrocellulose membrane and blocked and incubated with BHb (lanes 2–4). Subsequently, binding of Hb with purified proteins was detected by avidin-HRP. Total membrane fraction was used as a control (lane 1). D, determination of HbR in *Leishmania* as an integral membrane protein by analytical phase separation in nonionic detergent as described as described under "Experimental Procedures." Subsequently, the distribution of HbR in detergent (lane 1) and aqueous (lane 2) phases was determined by Western blot analysis using anti-HbR antibody. Results are representative of three independent preparations.

## RESULTS

**Purification of HbR from *Leishmania* Promastigote**—To purify the 46-kDa HbR from *Leishmania* promastigote membrane, the 46-kDa membrane protein was enriched by sequential ammonium sulfate precipitation (Fig. 1A, lane 4) and subsequently separated from other membrane proteins by ion-exchange chromatography. The results presented in Fig. 1B show that a ~46-kDa protein from the membrane protein-enriched fraction specifically eluted from the SP-Sepharose column at salt concentrations of ~140 to 190 mM (Fig. 1B, lanes 7–9). To determine the Hb binding ability of the purified 46-kDa protein eluted in fractions 5–7 (Fig. 1B, lanes 7–9), pro-

teins were transferred onto a nitrocellulose membrane, blocked with 5% bovine serum albumin, and incubated with BHb (10 ng/ $\mu$ l). Binding of Hb with purified proteins was detected by probing with avidin-HRP (1 ng/ $\mu$ l). The results presented in Fig. 1C demonstrate that the 46-kDa protein purified from *Leishmania* binds Hb.

The amphiphilic nature of the *Leishmania* HbR was investigated by analytical phase separation of membrane proteins in the nonionic detergent Triton X-114 (20). Subsequently, Western blot analysis was carried out to check the distribution of HbR in detergent phase using a specific antibody. The majority of HbR was recovered in the detergent phase as shown in Fig. 1D.

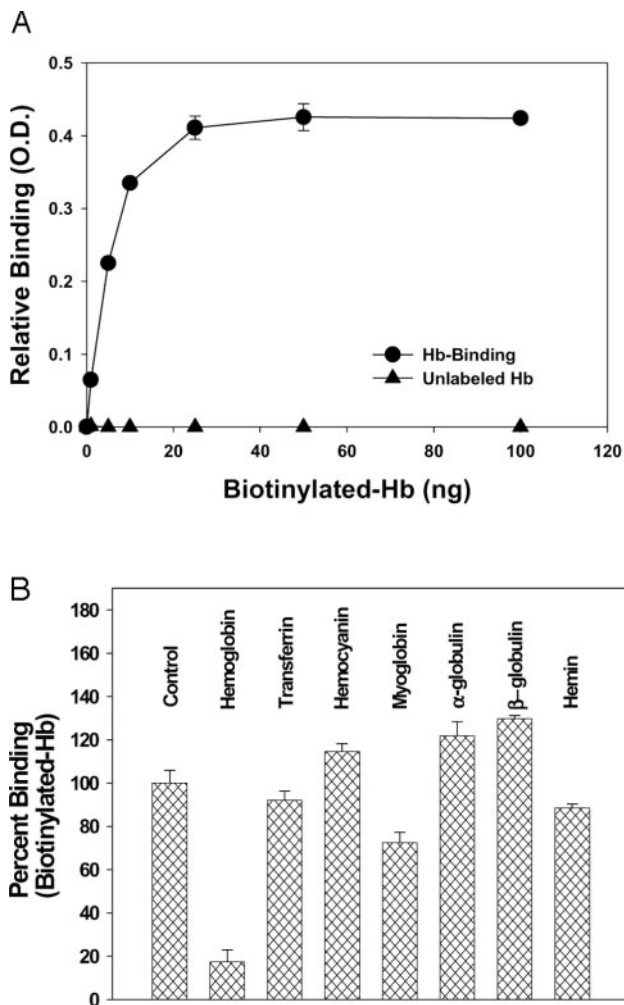


FIG. 2. Kinetics of Hb binding with purified protein. A, the purified protein was coated on an enzyme-linked immunosorbent assay plate, washed, and incubated with the indicated concentrations of BHb in the presence ( $\blacktriangle$ ) or absence ( $\bullet$ ) of unlabeled Hb as described as described under "Experimental Procedures." Finally, binding of BHb with purified protein was detected using avidin-HRP. The HRP activity present in each well was measured to quantitate the binding, and results are expressed as relative binding of three independent experiments  $\pm$  S.D. B, to determine the specificity, binding of BHb (25 ng) with immobilized HbR was carried out in the presence of a 100-fold excess of the indicated heme- or iron-containing proteins as described under "Experimental Procedures." The HRP activity measured in the absence of any competitor was taken as 100%, and results are expressed as a percentage of BHb bound of three independent experiments  $\pm$  S.D.

**Specific Binding of Hb with Purified HbR from *Leishmania***—To determine the binding of Hb to the purified protein, the protein was immobilized and incubated with increasing concentrations of BHb (0–1  $\mu$ g/ml) in 100  $\mu$ l of phosphate buffer (50 mM, pH 6.5). The binding of BHb with purified protein followed saturation kinetics, and maximum binding was observed with 50 ng of Hb (Fig. 2A). The half-maximal binding of BHb was achieved at 5 ng of the protein. Moreover, the binding of BHb with purified protein was effectively inhibited by unlabeled Hb (Fig. 2A), but not by various other heme- and iron-containing compounds, *viz.*, hemocyanin, transferrin,  $\alpha$ -globin,  $\beta$ -globin, and myoglobin (Fig. 2B). These results demonstrate that purified protein specifically binds Hb.

**Cloning and Expression of HbR from *Leishmania***—In order to clone HbR from *Leishmania*, the purified protein was subjected to tryptic digestion, and the peptides obtained were purified and sequenced by mass spectrometry (Fig. 3A). Subsequently, different sets of primers were designed based on the

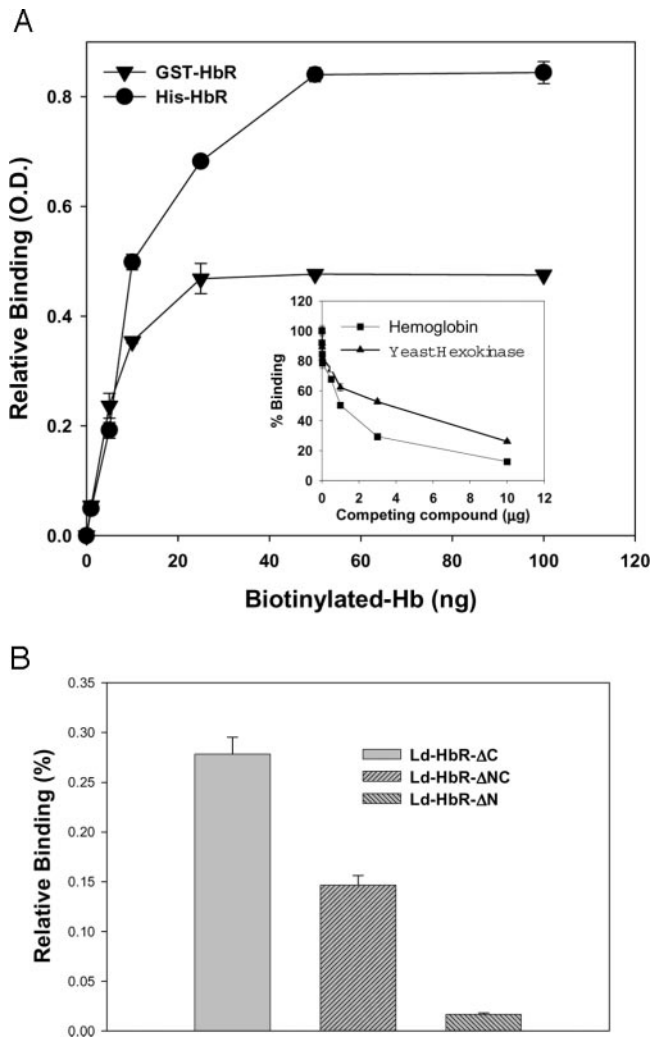
peptide sequences to amplify appropriate fragments from *Leishmania* cDNA. Thus, forward and reverse primers designed against peptides 17 and 14, respectively, were used to amplify a 482-bp internal fragment of HbR from *Leishmania* cDNA (Fig. 3B, lane 2). In *Leishmania*, the 5' end of all mature mRNA is known to contain a conserved 35-nucleotide spliced-leader sequence called the mini-exon, as a consequence of trans-splicing (27). Accordingly, using forward primer against the mini-exon and reverse primer against peptide 17, a 553-bp fragment corresponding to the 5' end of the gene was amplified from *Leishmania* cDNA (Fig. 3B, lane 3). Finally, a 893-bp fragment corresponding to the 3' end of the gene was amplified using a forward primer against peptide 14 and a reverse primer against the poly(A) tail by 3'-RACE (Fig. 3B, lane 4). Subsequently, putative start and stop codons were predicted from the 5'- and 3'-end fragments, respectively. Appropriate forward and reverse primers were designed and used to amplify the full-length (1416 bp) HbR gene from *L. donovani* cDNA (Fig. 3B, lane 5). The PCR product was cloned, sequenced, and hypothetically translated (471 amino acids). All peptides obtained from tryptic digestion of the purified native protein could be mapped into the cloned protein (Ld-HbR). Comparison of the Ld-HbR sequence with other HK sequences using ClustalW multiple sequence alignment (28) demonstrated the presence of conserved HK motifs in *Leishmania* HbR and revealed that the cloned protein has ~79% homology with *Trypanosoma*, 46% with *Entamoeba*, 52% with yeast, and 49% with human HK (Fig. 3C).

Accordingly, we have measured the HK activity of different Ld-HbR recombinant proteins (10  $\mu$ g), *e.g.* GST-HbR, His-HbR, and HbR-His, with tag at either the C or N terminus of the protein. The results presented in Fig. 3D demonstrate that purified recombinant HbR with tag at either the C or N terminus shows significant HK activity. However, the apparent increase of HK activity with His-tagged HbR in comparison with GST-HbR is due to the higher molar substitution of HbR in His-HbR form. This is supported by the fact that an equimolar amount (313 nmol) of the indicated recombinant proteins shows similar HK activity (Fig. 3D, inset).

**The N Terminus of HbR Is Required for Hb Binding**—The results presented in Fig. 4A show that both GST-HbR and His-HbR bind with Hb with similar kinetics, as observed with the protein purified from *Leishmania* promastigotes, and binding of Hb with GST-HbR is specifically competed by unlabeled Hb and commercially available yeast HK (Fig. 4A, inset). However, the apparent increase of Hb binding with His-HbR in comparison with GST-HbR is due to the higher molar substitution of HbR in His-HbR. In order to determine the Hb-binding region of the receptor, we have cloned and expressed different truncated forms of the receptor as GST fusion proteins: the N terminus (Ld-HbR- $\Delta$ C; amino acids 1–126), the middle region (Ld-HbR- $\Delta$ NC; amino acids 121–276), and the C terminus (Ld-HbR- $\Delta$ N; amino acids 270–471). Subsequently, these truncated proteins were used to determine their Hb binding ability. The results presented in Fig. 4B show that Hb predominantly binds to the N-terminal fragment (Ld-HbR- $\Delta$ C) in comparison with the C-terminal fragment (Ld-HbR- $\Delta$ N) of HbR. However, moderate binding of Hb was also observed with the middle fragment (Ld-HbR- $\Delta$ NC), suggesting that the Hb-binding site possibly straddles these two fragments.

**HbR from *Leishmania* Is a Hexokinase**—In order to determine whether the HbR in *Leishmania* is a HK, we transfected *Leishmania* with the HbR gene and measured total HK activity associated with cell lysate. Accordingly, full-length HbR was overexpressed in *Leishmania* promastigotes using pXG vector, and stable clones were selected in the presence of G418 anti-





**FIG. 4. Binding of Hb with recombinant HbR.** *A*, recombinant HbR was expressed as a GST or His tag fusion protein and immobilized onto an enzyme-linked immunosorbent assay plate. Hb binding was detected using BHB as described previously. The results are expressed as relative binding of three independent experiments  $\pm$  S.D. Binding of BHB (25 ng) with GST-HbR was also determined in the presence of different concentrations of Hb or yeast HK (*inset*). The HRP activity present in the absence of any competitor was taken as 100%, and results are expressed as a percentage of biotinylated hemoglobin bound of three independent experiments  $\pm$  S.D. *B*, deletion mutants HbR- $\Delta$ C, HbR- $\Delta$ NC, and HbR- $\Delta$ N, corresponding to the N terminus, middle region, and C terminus of HbR, respectively, were expressed and purified as GST fusion proteins. Proteins were immobilized, and Hb binding with each mutant was detected as described previously and expressed as relative binding of three independent experiments  $\pm$  S.D.

biotic. The overexpression of HbR in transfected cells in comparison with control cells was confirmed by Western blot analysis of the cell lysates using anti-HbR antibody (Fig. 5A, *inset*). Moreover, lysate prepared from cells overexpressing HbR showed significantly higher HK activity as compared with vector-transfected (control) *Leishmania* (Fig. 5A), demonstrating that HbR in *Leishmania* is a HK.

In order to further explore the possibility that the HK present on the cell surface of *Leishmania* is HbR, binding of  $^{125}\text{I}$ -Hb (6  $\mu\text{g}/\text{ml}$ ) by *Leishmania* at 4  $^{\circ}\text{C}$  was determined in the presence of antibodies raised against the recombinant N-terminal (Ld-HbR- $\Delta$ C, amino acids 1–126) and C-terminal (Ld-HbR- $\Delta$ N) fragments of HbR. Western blot analysis show that anti-Ld-HbR- $\Delta$ C antibody specifically recognized the N-terminal fragment (Ld-HbR- $\Delta$ C) and full-length protein (HbR), but not the C-terminal fragment (Fig. 5B, *inset*). The results presented in

the Fig. 5B shows that anti-Ld-HbR- $\Delta$ C specifically inhibits the binding of  $^{125}\text{I}$ -Hb, whereas no significant inhibition of binding was observed with anti-Ld-HbR- $\Delta$ N. Unlabeled Hb (600  $\mu\text{g}/\text{ml}$ ) was used as a positive control. Similarly, >50% of the uptake of  $^{125}\text{I}$ -Hb at 23  $^{\circ}\text{C}$  by *Leishmania* is inhibited specifically by full-length Ld-HbR and Ld-HbR- $\Delta$ C, but not by Ld-HbR- $\Delta$ N, further confirming the fact that the recombinant protein showing HK activity present on the *Leishmania* cell surface is involved in Hb binding through its N-terminal end (Fig. 5C).

**Localization of HbR on *Leishmania* Promastigotes**—Because the N-terminal of HbR is the extracellular Hb-binding domain of the receptor, a polyclonal antibody was raised against the Ld-HbR- $\Delta$ C fragment that specifically recognized a 46-kDa protein from *Leishmania* promastigote lysate (data not shown). In order to localize Ld-HbR on *Leishmania* promastigotes, cells were washed and incubated with anti-Ld-HbR- $\Delta$ C antibody at 4  $^{\circ}\text{C}$  to determine the surface component. Confocal images presented in Fig. 6 show that the HbR in *Leishmania* is localized above the nucleus (Fig. 6d) and at the base of the flagellum (Fig. 6h), indicating their localization in the flagellar pocket.

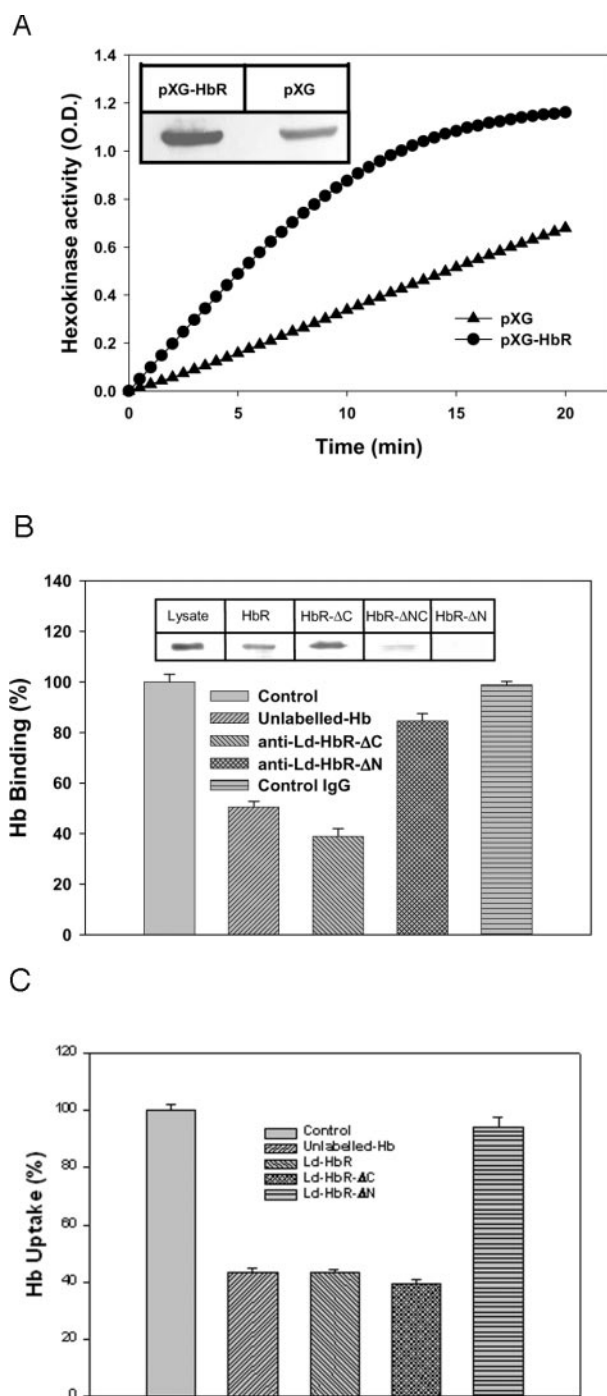
#### DISCUSSION

We have shown previously that endocytosis of Hb in *Leishmania* is mediated through a receptor present in the flagellar pocket (8), possibly to generate intracellular heme after transporting the internalized Hb to the late degradative compartment (9). These results suggest that *Leishmania* utilize the 46-kDa protein as a HbR to acquire heme through Hb endocytosis because these parasites lack a complete heme biosynthetic pathway (2).

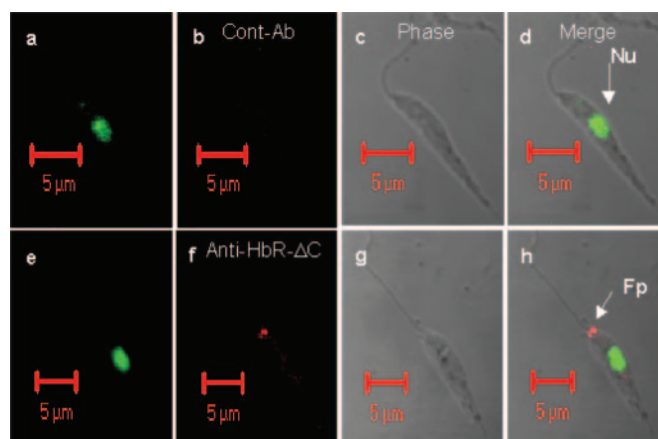
In the present study, the 46-kDa protein was purified from the solubilized membrane preparation of *Leishmania* by serial ammonium sulfate precipitations, followed by cation-exchange column chromatography (Fig. 1, A and B). The detection of HbR in the detergent phase in analytical phase separation with nonionic detergent indicated that it was an integral membrane protein (Fig. 1D). Moreover, localization of HbR in the flagellar pocket and similar Hb binding kinetics and specificity (Figs. 1C and 2, A and B) as observed previously with intact *Leishmania* promastigotes (8) demonstrated that the purified protein from *Leishmania* membrane is Hb receptor.

To determine the nature of HbR, we have cloned and expressed HbR from *Leishmania* as a GST fusion protein using primers designed against different peptide sequences obtained from tryptic digestion of the purified protein (Fig. 3A). Interestingly, the deduced amino acid sequence of the cloned HbR revealed (Fig. 3C) that Ld-HbR contains HK signature sequence  $^{156}\text{LGFTFSFPVDQKA VNKGLLIKWTQGF}^{281}$  (29), and ATP-binding motifs (30) such as  $^{88}\text{ALDLDGGTNFR}^{97}$ ,  $^{232}\text{VGVIIQTGSNAC}^{242}$ , and  $^{417}\text{AVDGSV}^{422}$ , in which the underlined residues represent conserved amino acids. Moreover, Ld-HbR also shows high homology with the recently described HK sequence from *Trypanosoma cruzi* (31) and contains PTS-II motif  $^4\text{RLXXLXXHI}^{12}$ , which is suggested to be responsible for targeting hexokinase to the glycosomes of the trypanosomatid parasites (21). These results, along with the finding that purified recombinant HbR with tag at either the C or N terminus shows significant HK activity (Fig. 3D), demonstrate that HbR in *Leishmania* is a HK.

The results presented in Fig. 4A show that Ld-HbR expressed as GST fusion protein specifically binds with Hb, and no binding was detected with free GST (data not shown). Moreover, the Hb binding ability of the different truncated mutants of the receptor showed that Hb-binding domain of the receptor resides in the N-terminal region of the protein (Fig. 4B). Binding of Hb with Ld-HbR was also competed by yeast HK, further substantiating the fact that HbR in *Leishmania* is probably a



**FIG. 5. Determination of HK activity of HbR.** A, HbR was overexpressed in *Leishmania* promastigotes as described under "Experimental Procedures." Overexpression was determined by Western blot analysis using specific antibody from respective cell lysate (inset). Subsequently, HK activity was measured from HbR-overexpressing (●) and vector-transfected (▲) cell lysates (10  $\mu$ g) as described under "Experimental Procedures." Results are representative of three independent preparations. B, promastigotes ( $1 \times 10^7$  cells/ml) were incubated with 6  $\mu$ g/ml  $^{125}$ I-Hb in the absence or presence of the indicated antibody at 4 °C for 2 h as described under "Experimental Procedures." Hb was used as control. Results are expressed as the percentage of Hb bound by the cells in the absence of any competitor (100% bound) and represent an average of three determinations  $\pm$  S.D. The specificity of anti-Ld-HbR- $\Delta$ C antibody was determined by Western blot analysis using the indicated form of HbR (inset). C, to determine the uptake, cells ( $1 \times 10^7$  cells/ml) were incubated with 1  $\mu$ g/ml  $^{125}$ I-Hb in the absence or presence of the same concentration of different fragments of HbR at 23 °C for 4 h as described under "Experimental Procedures." Hb (100  $\mu$ g/ml) was used as control. Results are expressed as the percentage of Hb bound by the cells in the absence of any competitor (100% bound) and represent an average of three determinations  $\pm$  S.D.



**FIG. 6. Confocal images showing the localization of HbR on *L. donovani* promastigotes.** Cells were incubated with anti-Ld-HbR- $\Delta$ C antibody for 1 h at 4 °C and subsequently probed with Texas Red-conjugated goat anti-mouse secondary antibody (f and h) as described under "Experimental Procedures." Pre-immune serum was used as control (b and d). Promastigotes were pre-incubated with SYTO-14 to label the nucleus (a, d, e, and h). Nu, nucleus; Fp, flagellar pocket. c and g are phase images.

HK. However, we were unable to detect HK activity in the purified endogenous protein (Fig. 3D), possibly due to the use of detergent and urea in the purification procedure. Recent studies have shown that even His-tag containing recombinant HK displays less affinity for its substrate than the natural protein (31), possibly because of the additional N-terminal tag in the recombinant protein that presumably hinders the native confirmation needed for HK activity. To rule out the interference of the tag as well as to detect the enzymatic activity of Ld-HbR in the native environment of *Leishmania* promastigotes, we overexpressed the untagged HbR in *Leishmania* and measured the total HK activity associated with the lysate. The consistently higher HK activity of HbR-overexpressing cells in comparison with control cells indicates that the hemoglobin receptor in *Leishmania* is a HK (Fig. 5A). HK activity measured from 10  $\mu$ g of HbR-overexpressing cell lysate corresponds to the HK activity seen with 10  $\mu$ g of purified recombinant protein, demonstrating that native conformation is needed for proper HK activity. Moreover, binding of  $^{125}$ I-Hb with the receptor present on the surface of *Leishmania* is significantly blocked by anti-Ld-HbR- $\Delta$ C, demonstrating that HK present on the surface of *Leishmania* is involved in Hb binding (Fig. 5B). Furthermore, inhibition of uptake of  $^{125}$ I-Hb in *Leishmania* by Ld-HbR and Ld-HbR- $\Delta$ C, but not by Ld-HbR- $\Delta$ N (Fig. 5C), further strengthened the observation that cell surface-localized HK is HbR in *Leishmania* that binds with Hb through its N-terminal extracellular domain. These observations are consistent with our previous results that C-terminal is the cytoplasmic domain of HbR (9).

In contrast to mammalian cells, endocytosis in trypanosomatids occurs exclusively through the flagellar pocket (32). Immunolocalization studies using anti-Ld-HbR- $\Delta$ C antibody indicated that HbR is localized in the flagellar pocket of *Leishmania* promastigotes (Fig. 6). These results are in agreement with our earlier studies, in which we have shown that Hb binds with *Leishmania* promastigotes in the flagellar pocket (8). Thus, the observation that Ld-HbR, which localized on the cell surface, has HK activity and Hb binding ability conclusively proves that Hb receptor in *Leishmania* is HK present in the flagellar pocket.

This investigation identifies a form of HK, a glycolytic enzyme, localized in the flagellar pocket on the cell surface and performing as an endocytic receptor for internalization of Hb in

*Leishmania* and adds to the small but growing list of glycolytic enzymes of essentially identical primary structures performing diverse functions at different locales of the same cell or cell types (33). For instance, cell surface-associated glyceraldehyde-3-phosphate dehydrogenase was shown to be a fibronectin and laminin-binding protein in *Candida albicans* (34) and involved in iron acquisition in *Staphylococcus* (35). Moreover, glyceraldehyde-3-phosphate dehydrogenase also plays major role in endoplasmic reticulum to Golgi transport by regulating vesicle fusion (36, 37). In concordance with our observation, a 95-kDa phosphotyrosine-containing protein, localized on the surface of mouse spermatozoa and serving as a cell surface receptor for ZP3 (38), was identified as HK (39). Recent reports also suggest that the HK in *Arabidopsis* not only catalyzes the ATP-dependent phosphorylation of glucose but also senses glucose levels and the phosphorylation status of glucose and transmits these signals to the nucleus through a signal transduction pathway (40). Similarly, HK present on the chloroplast outer envelope regulates glucose export by phosphorylating glucose before it enters the cytosol (41). It will be interesting to determine whether similar phosphorylation by HbR plays any role in the import of Hb by *Leishmania*.

In conclusion, our results represent the first documentation that Hb endocytosis in *Leishmania* is mediated through a HK present in the flagellar pocket. The ability of HK to bind Hb has not been observed in any other system; however, under certain conditions, Hb is found to be associated with type II HK present in the erythrocyte of newborn infants (42). Hence, it is possible that this unusual binding capacity is an inherent characteristic of HK and might have evolved more significantly in hemoparasites such as *Leishmania* because of their imperative requirement of heme. However, how glycolytic enzymes such as HK are exported to the cell surface is a very intriguing question and is currently being investigated.

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