

Hemoglobin Endocytosis in *Leishmania* Is Mediated through a 46-kDa Protein Located in the Flagellar Pocket*

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Four lines of evidence indicate that a specific high affinity binding site on the surface of *Leishmania donovani* promastigotes mediates rapid internalization and degradation of hemoglobin. 1) Binding and uptake of ¹²⁵I-hemoglobin by *Leishmania* followed saturation kinetics and were competed by unlabeled hemoglobin but not by globin or hemin or other heme- or iron-containing proteins. 2) Immunogold labeling studies revealed that, at 4 °C, hemoglobin binding was localized in the flagellar pocket of the promastigotes. Indirect immunofluorescence assays showed that, at 37 °C, the bound hemoglobin in such cells entered an endocytic compartment within 2 min and dispersed throughout the cell body by 15 min. 3) After incubation with hemoglobin-gold conjugates at 25 °C or 37 °C, the particles accumulated in discrete intracellular vesicles. 4) A single biotinylated protein of 46 kDa was revealed when solubilized membranes from surface biotinylated intact *Leishmania* adsorbed by hemoglobin-agarose beads were subjected to SDS-polyacrylamide gel electrophoresis and Western blotting with avidin-horseradish peroxidase. Considered together, these data indicate that this 46-kDa protein on the cell surface of *L. donovani* promastigotes mediates the binding of hemoglobin and its rapid internalization through a vesicular pathway characteristic of receptor-mediated endocytosis.

In both eukaryotic and prokaryotic cells, endocytosis is essential for uptake of nutrients, down-regulation of cell surface receptors, and maintenance of cell homeostasis (1–3). Among the protozoans, endocytosis has been widely studied in trypanosomatids like *Trypanosoma* and *Leishmania* (4–6). These hemoflagellates are found predominantly in the blood stream and tissues of mammalian host, where they rapidly multiply. Receptor systems have been identified on trypanosomatid parasites, which mediate uptake of low density lipoprotein and transferrin presumably for efficient supply of nutrients required for rapid growth such as cholesterol and metal ions, which usually occur as tightly bound complexes with carrier proteins (7, 8).

Leishmania sp. are protozoan parasites responsible for several diseases varying from a single cutaneous lesion to fatal visceral leishmaniasis, affecting millions of people worldwide

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(9). *Leishmania donovani* is the etiologic agent of kala-azar, a chronic and often fatal form of human visceral leishmaniasis (10).

In common with most trypanosomatids, *Leishmania* lack a complete heme biosynthetic pathway and must therefore acquire heme from external sources (11–13). In the animal host, heme is present mainly as hemopexin and hemoglobin of which hemoglobin is the most important reservoir (14). This potential source may be available when erythrocytes are lysed by hemolysins or natural degradation of hemoglobin in macrophages or by hitherto unknown mechanisms. Galbraith *et al.* (15) have reported the presence of specific heme-binding sites on *Leishmania mexicana amazonensis* promastigotes. Since *Leishmania* promastigotes can also be grown *in vitro* in blood agar medium without addition of heme (16), it is possible that *Leishmania* might have evolved mechanisms to internalize intact hemoglobin and generate heme intracellularly. Hemoglobin-binding proteins have been identified in some pathogens, suggesting that selective recognition of hemoglobin occurred at the cell surface (17–21). However, no specific hemoglobin-binding site has so far been reported to be present on *Leishmania*.

In the present investigation, we have shown that a limited number of sites localized in the flagellar pocket of *L. donovani* promastigotes mediate uptake and degradation of hemoglobin with saturation kinetics. Furthermore, we also report the presence of a 46-kDa protein on the cell surface of *L. donovani* promastigotes that specifically binds hemoglobin.

EXPERIMENTAL PROCEDURES

Reagents—Hemoglobin, hemin, globin, transferrin, hemocyanin, myoglobin, colloidal gold particles, rabbit anti-hemoglobin antibodies, and hemoglobin-agarose affinity matrix were purchased from Sigma. Goat anti-rabbit IgG conjugated with 20-nm colloidal gold was purchased from Jackson ImmunoResearch Laboratory, West Grove, PA. *N*-Hydroxysuccinimido-biotin, avidin-horseradish peroxidase (avidin-HRP),¹ and biconchonic acid reagents were purchased from Pierce. Anti-hemoglobin antibody in mice was raised by standard technique (22). FITC-labeled horse anti-mouse IgG was purchased from Vector Laboratories, Burlingame, CA. Other reagents used were of analytical grade.

Leishmania—Promastigotes of *L. donovani* (UR 6) were obtained from the Indian Institute of Chemical Biology, Calcutta, India. The cells were routinely maintained on solid blood agar slants containing glucose, peptone, sodium chloride, beef heart extract, and rabbit blood with gentamycin (23, 24). For experimental purposes, cells were harvested from 3-day-old blood agar slants by scraping into phosphate-buffered (10 mM, pH 7.2) saline (0.15 M).

Radioiodination of Hemoglobin—Hemoglobin was labeled with Na¹²⁵I by the iodine monochloride-catalyzed reaction (2). More than 98% of the radioactivity was acid precipitable, and the specific activity varied between 100 and 200 cpm/ng of hemoglobin from batch to batch.

¹ The abbreviations used are: HRP, horseradish peroxidase; BSA, bovine serum albumin; PBS, phosphate-buffered saline; FITC, fluorescein isothiocyanate; PAGE, polyacrylamide gel electrophoresis.

Binding of ^{125}I -Hemoglobin to *L. donovani* Promastigotes at 4 °C—Promastigotes were suspended in RPMI medium and washed three times with the same media. Cells (5×10^7) were resuspended in RPMI containing 1 mg/ml bovine serum albumin (BSA) and incubated at 4 °C with varying concentrations of ^{125}I -hemoglobin. After 4 h, the cells were washed three times with PBS containing 1 mg/ml BSA and then three times with PBS to remove unbound radioactivity. The cell pellet was finally dissolved in 0.1 N NaOH. The cell-associated radioactivity was then measured by placing an aliquot in a γ counter. Cellular protein content was estimated by bicinchoninic acid reagent (25). The results were expressed as femtomoles of hemoglobin bound/ μg of cell protein. To determine the specificity, binding of ^{125}I -hemoglobin (6 $\mu\text{g}/\text{ml}$) with promastigotes was carried out in the presence of different competitors as indicated.

Uptake and Degradation of ^{125}I -Hemoglobin by *Leishmania* at 37 °C—Cells (5×10^7) were incubated with increasing concentrations of ^{125}I -hemoglobin in 1 ml of RPMI 1640 medium containing 1 mg/ml BSA at 37 °C. After 3 h the cells were pelleted by centrifugation at $500 \times g$ for 10 min. Aliquots of the supernatant were processed for the determination of trichloroacetic acid-soluble non-iodide radioactivity after extraction with chloroform (2). Thin layer chromatographic analysis showed that about 80% of the trichloroacetic acid-soluble radioactivity released into the medium consisted of monoiodotyrosines. The cells were washed as indicated in the binding experiment, and an aliquot was measured to determine the cell-associated radioactivity.

Electron Microscopy—*Leishmania* cells (5×10^5) were incubated with 6 $\mu\text{g}/\text{ml}$ hemoglobin for 2 h at 4 °C in RPMI 1640 medium containing 1 mg/ml BSA. The cells were washed five times with ice-cold PBS to remove unbound hemoglobin and fixed in 1% glutaraldehyde and 1% paraformaldehyde in PBS, pH 7.2, for 20 min at 4 °C. Subsequently, the cells were washed, dehydrated in ethanol, and embedded in LR White resin. Ultrathin sections of the LR White-embedded cells were blocked overnight with 2.5% casein in 0.001% Tween 20 in PBS. Sections were washed five times with PBS-Tween 20 and incubated with rabbit anti-hemoglobin antibody (1:100) for 2 h at room temperature. Sections were washed five times in similar manner (5 min each time), and they were incubated with goat anti-rabbit IgG conjugated with 20-nm colloidal gold (1:10) for 1 h at 37 °C to allow the detection of primary antibody binding. Finally, the sections were stained with uranyl acetate and viewed at 80 kV in a transmission electron microscope (Joel 1200 EX 11). Cells without hemoglobin bound was used as control.

To determine the intracellular location of hemoglobin inside the *Leishmania*, 5×10^5 cells were incubated with gold particles (20 nm) conjugated with hemoglobin (1 μg of hemoglobin/ml) as described by De Mey (26) for 1 h at 25 °C or at 37 °C in RPMI 1640 medium containing 1 mg/ml BSA. The cells were washed five times with cold PBS to remove unbound gold and fixed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.3, washed, and treated with 1% OsO₄ in 0.1 M sodium cacodylate buffer pH 7.3. The cells were rinsed and dehydrated in ethanol and embedded in araldite (27). Thin sections were double-stained with uranyl acetate and lead citrate and examined at 60 kV with an electron microscope. BSA-gold was used as control. Finally, quantitation was carried out by counting the number of gold particles internalized by *Leishmania* for each set of experimental conditions.

Immunofluorescence Microscopy—*L. donovani* promastigotes (5×10^7) were washed three times with PBS, incubated with 5 $\mu\text{g}/\text{ml}$ hemoglobin for 2 h at 4 °C, washed, and incubated for different periods at 37 °C to allow internalization of the hemoglobin bound at 4 °C. Finally, the cells were washed and processed as described for the binding experiment.

Cells were resuspended in PBS, and thin smears of cells were prepared on glass slides. Subsequently, the cells were fixed in precooled (−20 °C) methanol for 20 min (28). After fixation, cells were washed three times in PBS containing 0.2% Triton X-100 with 5 min of incubation at room temperature each time. Cells were then incubated with mouse anti-hemoglobin primary antibody in PBS for 1 h at 37 °C. After washing the cells three times with PBS (5 min/each), they were incubated with horse anti-mouse FITC in PBS for 1 h at 37 °C to allow the detection of primary antibody binding. Cells were then washed once with PBS containing 0.2% Triton X-100 and three times with PBS (5 min/each). The slides were then mounted in glycerol containing para-phenylenediamine and viewed in a fluorescence microscope (Olympus).

Biotinylation of *Leishmania* Surface Proteins—Cell surface proteins of intact *L. donovani* promastigotes were biotinylated using standard procedure (29). Briefly, cells were washed and incubated with 500 $\mu\text{g}/\text{ml}$ N-hydroxysuccinimido-biotin for 1 h at 4 °C. Subsequently, the cells were washed three times with PBS containing 50 mM NH₄Cl to remove excess biotin, followed by one wash with PBS.

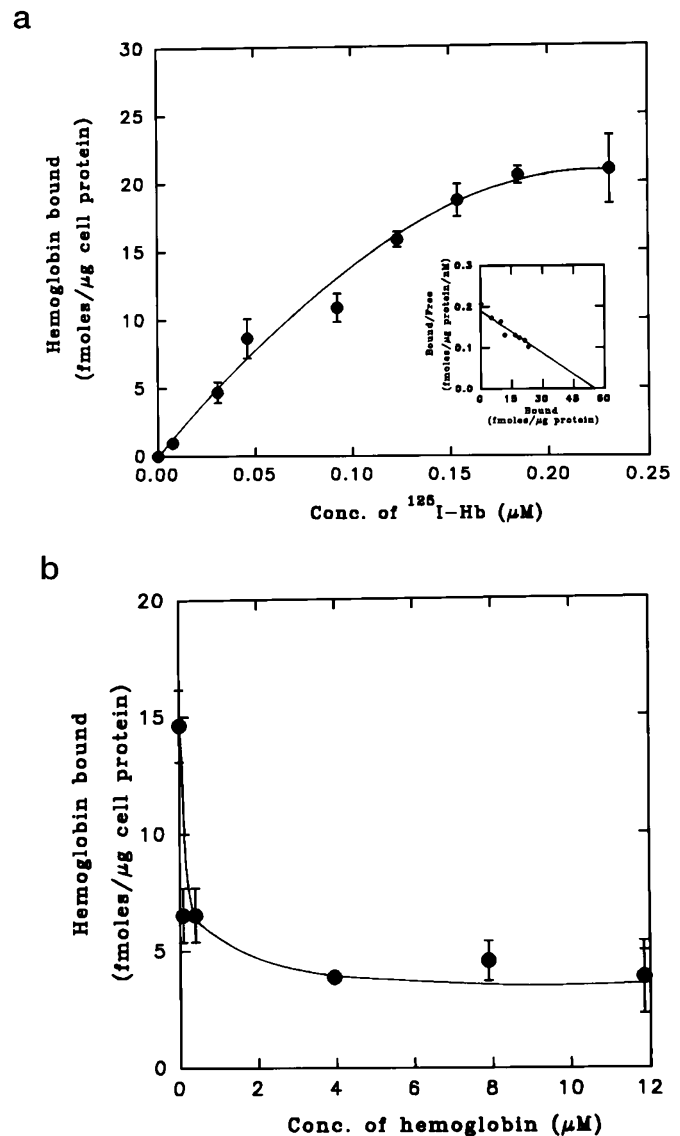


FIG. 1. *a*, binding of ^{125}I -hemoglobin to *Leishmania donovani* promastigotes at 4 °C. Promastigotes ($5 \times 10^7/\text{ml}$) were incubated with ^{125}I -hemoglobin at 4 °C for 4 h and processed as described under "Experimental Procedures." Data, expressed as femtomoles of labeled protein bound/ μg of cellular protein, represent an average of three determinations \pm S.E. *Inset* shows the Scatchard plot of the binding data. K_d value as determined from the slope was found to be 0.25 μM while the number of sites per promastigote was derived by dividing the product of the abscissa and Avogadro's number by the number of cells taken per milliliter. *b*, competition by unlabeled hemoglobin for binding of ^{125}I -hemoglobin by *Leishmania*. Promastigotes ($5 \times 10^7/\text{ml}$) were incubated with ^{125}I -hemoglobin (0.09 μM) and indicated concentrations of unlabeled hemoglobin for 4 h at 4 °C. Data represent an average of three determinations \pm S.E.

Preparation of Membrane Fraction—Biotinylated *L. donovani* promastigotes were washed three times in PBS and then kept for 1 h at 4 °C in hypotonic Tris-HCl buffer (5 mM, pH 7.2). Then, the cells were broken by sonication (3×20 s) and the unbroken cells and nucleus were separated by low speed centrifugation ($200 \times g$ for 10 min.). The resultant postnuclear supernatant was again centrifuged at $100,000 \times g$ for 1 h. The membrane pellet was treated with PBS containing 2% β -octyl glucoside and kept at 4 °C overnight. Finally, extracted membrane proteins were separated from the debris by centrifugation at $100,000 \times g$ for 1 h at 4 °C. The supernatant thus obtained contained biotinylated membrane proteins.

Affinity Chromatography of the Biotinylated Membrane Proteins—The extract (100 μl) containing biotinylated membrane proteins (0.6 mg) was added to hemoglobin-agarose beads (100 μl of packed gel containing 1.6 mg of hemoglobin) equilibrated with 10 mM phosphate

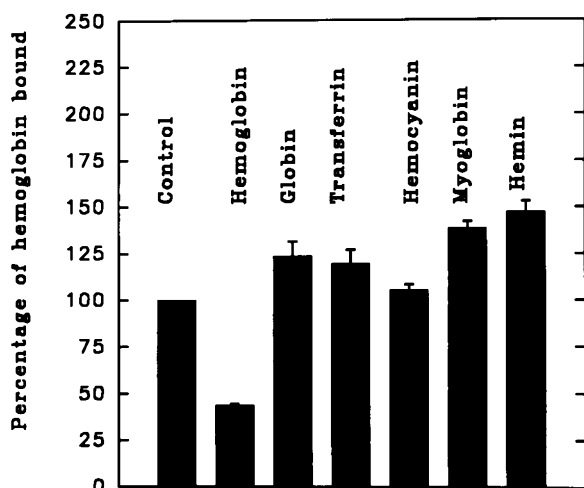


FIG. 2. Effect of various compounds on the binding of ^{125}I -hemoglobin at 4 °C. Promastigotes ($5 \times 10^7/\text{ml}$) were incubated with $0.09 \mu\text{M}$ labeled hemoglobin in the absence or presence of 10-fold excess of the indicated compounds at 4 °C for 4 h. Data, expressed as percentage of hemoglobin bound by the cells in the absence of any competitor (100% bound), represent an average of three determinations \pm S.E.

buffer containing 1 M NaCl in a microcentrifuge tube and incubated overnight at 4 °C. The beads were then washed five times with the same buffer to remove the proteins bound nonspecifically with the affinity matrix. Subsequently, 30 μl of sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer (0.0625 M Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 0.002% bromphenol blue) was added to the washed beads and kept in a boiling water bath at 100 °C for 5 min. The sample was allowed to cool, and then the affinity beads were pelleted by centrifugation at $10,000 \times g$ for 5 min. The supernatant was transferred to a clean microcentrifuge tube, and 20 μl of the sample was subsequently applied to a 10% SDS-polyacrylamide gel for analysis by SDS-PAGE. Finally, proteins were transferred onto nitrocellulose membranes and Western blot analysis was carried out to detect the biotinylated surface proteins of *Leishmania* using avidin-HRP and developed by α -chloronaphthol.

RESULTS

Binding of ^{125}I -Hemoglobin at 4 °C—The data presented in Fig. 1a show the amount of ^{125}I -hemoglobin binding at 4 °C to the cells as a function of the concentration of ^{125}I -hemoglobin in the incubation medium. Half-maximal binding of ^{125}I -hemoglobin occurred at a concentration of about $0.09 \mu\text{M}$ hemoglobin (6 $\mu\text{g}/\text{ml}$). Scatchard plot (30) of the data in Fig. 1a exhibited a straight line ($K_d = 0.25 \mu\text{M}$). The number of hemoglobin binding sites per promastigote was found to be 1.2×10^5 . The binding of ^{125}I -hemoglobin to *Leishmania* was effectively inhibited by unlabeled hemoglobin (Fig. 1b) with 50% inhibition achieved at about $0.09 \mu\text{M}$ (6 $\mu\text{g}/\text{ml}$) hemoglobin. When cells were incubated with $0.09 \mu\text{M}$ (6 $\mu\text{g}/\text{ml}$) of ^{125}I -hemoglobin at 4 °C along with 10-fold excess of hemocyanin, transferrin, hemin, globin, and myoglobin, the binding of ^{125}I -hemoglobin was not affected (Fig. 2), indicating the specificity of these binding sites for hemoglobin.

Uptake and Degradation of ^{125}I -Hemoglobin at 37 °C—When promastigotes were incubated with different concentrations of ^{125}I -hemoglobin at 37 °C for 3 h, cell-associated radioactivity increased in a saturable fashion while a part of the added radioactivity was released into the medium as trichloroacetic acid-soluble material (Fig. 3a). The half-maximal value for uptake and degradation was achieved at about $0.065 \mu\text{M}$ hemoglobin. The data in Fig. 3b show, that when the promastigotes were incubated with varying concentrations of ^{125}I -hemoglobin at the optimal growth temperature of 25 °C, the content of cell-associated radioactivity reached saturation with a half-maximal value of $0.06 \mu\text{M}$ indicating expression of hemoglobin-

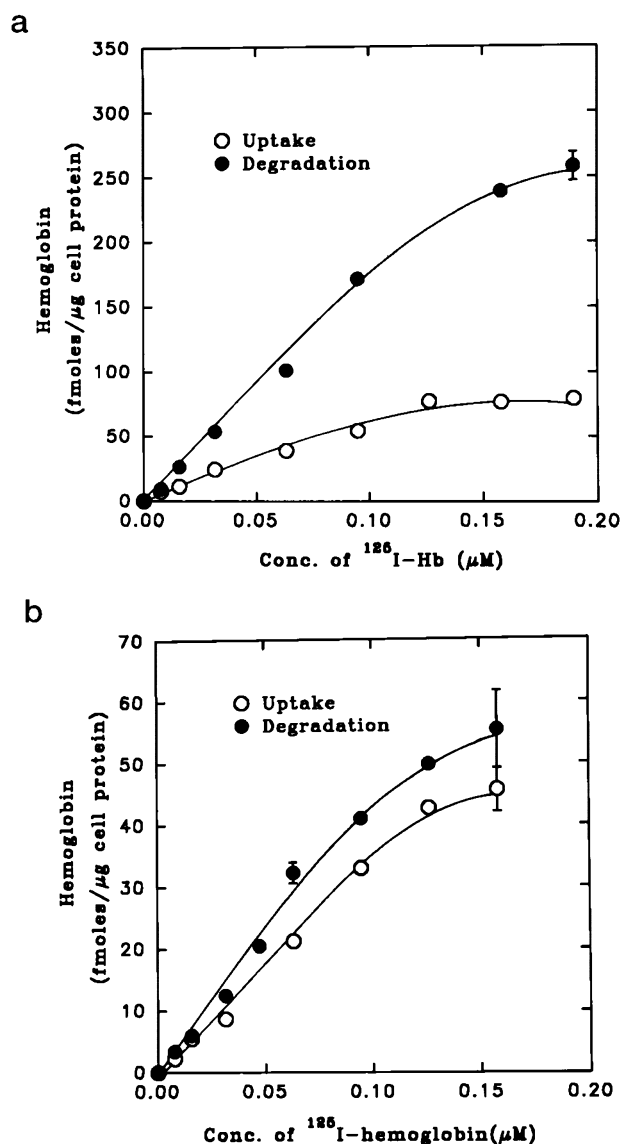


FIG. 3. Concentration dependence of uptake and degradation of ^{125}I -hemoglobin by *L. donovani* promastigotes at 37 °C or 25 °C. Promastigotes ($5 \times 10^7/\text{ml}$) were incubated with indicated concentrations of ^{125}I -hemoglobin at 37 °C (a) or 25 °C (b). After incubation for 3 h, the amounts of radioactivity accumulated in the cells and the trichloroacetic acid-soluble (non-iodide) degradation products of the labeled protein released in the medium were determined as described under "Experimental Procedures." Data, expressed as femtomoles of labeled protein taken up or degraded/ μg of cellular protein, represent an average of three determinations \pm S.E.

binding sites of similar affinity and number as at 37 °C. The amount of trichloroacetic acid-soluble radioactivity released in the medium, however, was about one-fifth of that at 37 °C. These results indicate that, even at 25 °C, *Leishmania* takes up hemoglobin and then degrades it, albeit to a lesser extent than at 37 °C. When *Leishmania* promastigotes were incubated with ^{125}I -hemoglobin ($0.09 \mu\text{M}$) at 37 °C for different time periods, cellular radioactivity reached a steady state plateau within 30 min while trichloroacetic acid-soluble radioactivity continued to increase at a linear rate (Fig. 4), indicating the simultaneous uptake and degradation of ^{125}I -hemoglobin.

Uptake of ^{125}I -hemoglobin at 37 °C was inhibited by unlabeled hemoglobin, whereas globin and hemin did not significantly inhibit the uptake of hemoglobin, indicating that the binding sites recognized hemoglobin as a whole (Fig. 5a). Unlabeled hemoglobin, but not globin, competed for the degrada-

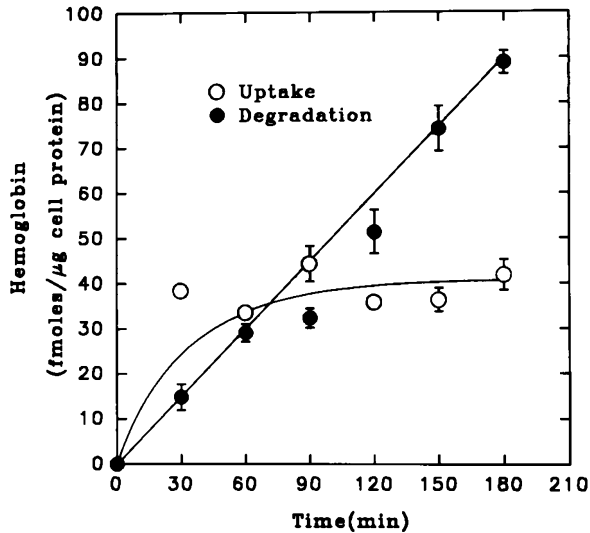


FIG. 4. Time course of uptake and degradation of ^{125}I -hemoglobin at 37°C . Promastigotes ($5 \times 10^7/\text{ml}$) were incubated with $0.09 \mu\text{M}$ ^{125}I -hemoglobin at 37°C . After indicated time intervals, cell-associated radioactivity and trichloroacetic acid-soluble non-iodide products of ^{125}I -hemoglobin degradation released in the medium were determined. Data represent an average of three determinations \pm S.E.

tion of ^{125}I -hemoglobin (Fig. 5b). Surprisingly, about 80% of the ^{125}I -hemoglobin degradation was inhibited by $45 \mu\text{M}$ hemin (Fig. 5b). We also noted that hemin ($15 \mu\text{M}$) inhibited the scavenger receptor-mediated degradation of maleylated bovine serum albumin in a macrophage cell line (data not shown). In contrast, hemoglobin degradation by *Leishmania* could not be inhibited by lysosomotropic agents chloroquine, monensin, or ammonium chloride (data not shown), which are potent inhibitors of lysosomal degradative processes in mammalian cells.

Internalization of Hemoglobin by *Leishmania*—When *Leishmania* previously incubated with hemoglobin at 4°C were treated with a rabbit anti-hemoglobin antibody followed by an anti-rabbit IgG conjugated with colloidal gold, accumulation of gold particles could be seen in the flagellar pocket (Fig. 6, b and e). In contrast, no gold particles were detected in the cells not incubated with hemoglobin (Fig. 6, a and d) or anti-hemoglobin antibody (Fig. 6, c and f).

To explore the fate of hemoglobin bound with high affinity to the sites in the flagellar pocket, we incubated the *Leishmania* with hemoglobin-gold for 1 h either at 37°C (Fig. 7, a and b) or at 25°C (Fig. 7, c and d) and localized the gold particles by electron microscopy. The results presented in Fig. 7 (a and c) show that the gold particles are localized in discrete intracellular vesicles, suggesting the internalization of hemoglobin at both temperatures. This result also indicated that transport of hemoglobin in *Leishmania* is mediated through vesicle fusion. The uptake of hemoglobin-gold particles was inhibited by incubating the cells with free hemoglobin in similar condition at both temperatures (Fig. 7, b and d), suggesting that uptake is mediated through specific binding sites as evidenced in the biochemical assay. In order to quantitate the uptake of hemoglobin-gold by *Leishmania*, we counted the number of gold particles internalized by 50 cells. The data in Table I show that *Leishmania* internalized about 371 gold particles in discrete vesicular structures when the cells were incubated at 37°C with hemoglobin-gold. In contrast, only 86 particles were detected when the *Leishmania* were incubated with the same concentration of hemoglobin-gold in presence of excess unlabeled hemoglobin. The uptake of BSA-gold, presumably through nonspecific fluid phase endocytosis, was about 10% of that of hemoglobin-gold. Similar results were also obtained

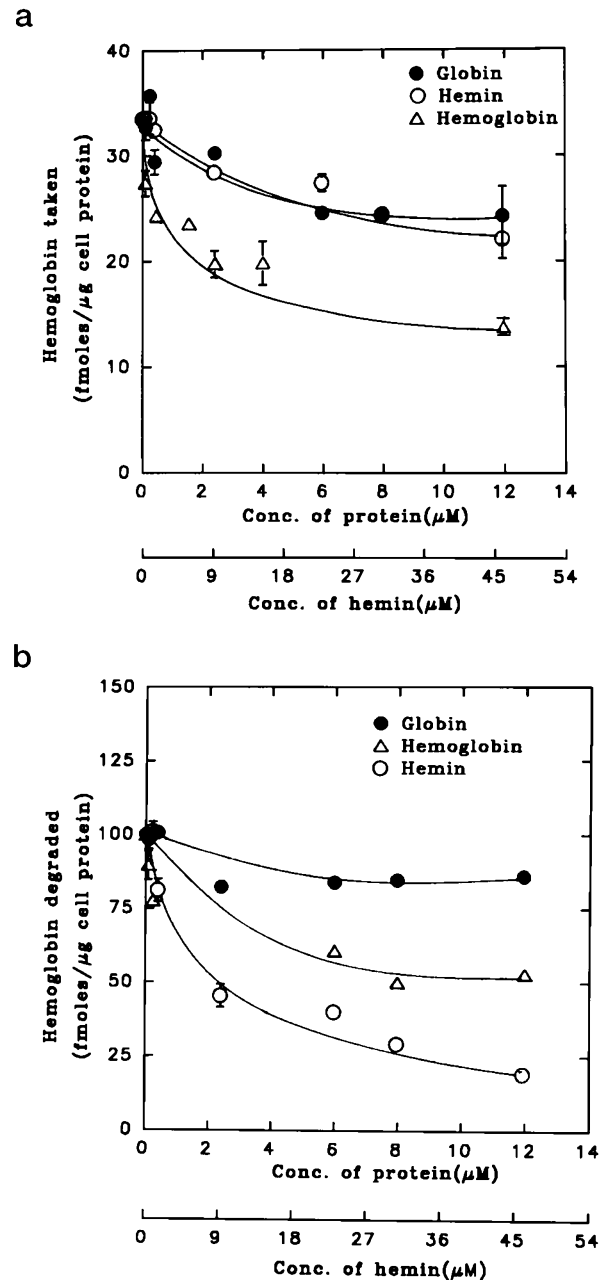


FIG. 5. Effect of hemin, globin, and unlabeled hemoglobin on the uptake and degradation of ^{125}I -hemoglobin by *L. donovani* promastigotes. Promastigotes ($5 \times 10^7/\text{ml}$) were incubated with $0.09 \mu\text{M}$ ^{125}I -hemoglobin at 37°C in presence of indicated concentrations of unlabeled hemoglobin, hemin and globin. The cell-associated radioactivity (a) and trichloroacetic acid-soluble degradation products (b) were expressed as femtomoles of protein taken up or degraded/ μg of cellular protein. Results are an average of three determinations \pm S.E.

when the same experiments were carried out at 25°C (Table I).

To follow the kinetics of hemoglobin uptake, after binding hemoglobin at 4°C the washed cells were incubated for different periods of time at 37°C , treated with anti-hemoglobin antibodies, and visualized by subsequent probing with secondary antibodies labeled with FITC. Immediately after binding at 4°C hemoglobin was visualized as a bright fluorescent spot (Fig. 8a) in the apical region of *Leishmania*. After warming to 37°C , progressive dispersal of the staining pattern began by 2 min, and by 15 min the fluorescence dispersed throughout the cell body (Fig. 8d).

Identification of Putative Hemoglobin-binding Proteins on *Leishmania*—In order to identify the putative hemoglobin-

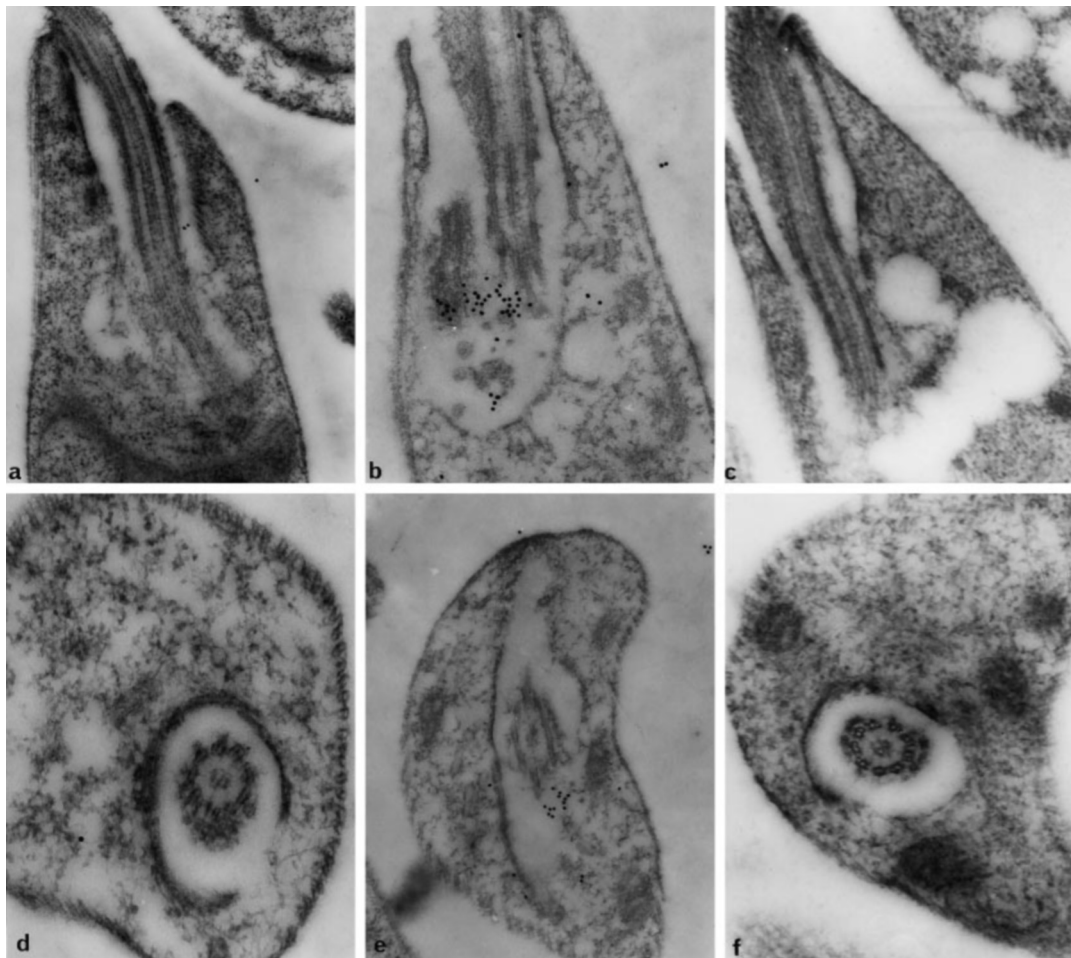


FIG. 6. Localization of hemoglobin in the flagellar pocket by immunogold labeling. *Leishmania* promastigotes (5×10^5) were incubated with (b, c, e, and f) or without (a and d) 6 $\mu\text{g}/\text{ml}$ hemoglobin for 2 h at 4 °C. The cells were washed to remove the unbound hemoglobin, fixed, and processed for immunogold labeling as described under "Experimental Procedures." Panels c and f show cells incubated with hemoglobin but without primary anti-hemoglobin antibody. Panels a, b, and c represent the longitudinal section of the flagellar pocket, and panels d, e, and f represent the cross-section of the flagellar pocket. Original magnification, $\times 50,000$ (a–c), $\times 70,000$ (d and f), and $\times 40,000$ (e).

binding sites, surface membrane fractions prepared from intact biotinylated promastigotes were solubilized with detergent and subjected to affinity chromatography on hemoglobin-agarose beads. The proteins from the *Leishmania* surface membranes bound to hemoglobin-agarose affinity matrix were separated by SDS-PAGE, and the biotinylated proteins were detected by Western blot analysis using avidin-HRP, which revealed a single band with a molecular mass of 46 kDa (Fig. 9, Control). Preincubation of the membrane preparation with hemoglobin effectively blocked the adsorption of the 46-kDa protein to hemoglobin-agarose beads, as can be seen by the reduced intensity of the band in the middle lane. In contrast, preincubation with hemin did not affect the binding of the protein to hemoglobin-agarose (last lane). Therefore, the 46-kDa band is likely to be the putative hemoglobin-binding protein on *Leishmania* surface.

DISCUSSION

Our study demonstrates absorptive endocytosis of hemoglobin by *L. donovani* promastigotes *in vitro*. At 4 °C ^{125}I -hemoglobin binds to *Leishmania* in a saturable manner, indicating that it is recognized by a limited number of binding sites on the cell surface (Fig. 1a). The binding sites recognize hemoglobin as a whole, which was evident from the fact that, although unlabeled hemoglobin effectively competed for the binding of ^{125}I -hemoglobin, free hemin and globin (Figs. 1b and 2), as well as the heme-containing α and β chains of hemoglobin (data not

shown) did not inhibit the binding. The exquisite specificity of these binding sites for the particular conformation of intact hemoglobin molecule is further highlighted by the fact that hemoglobin binding to *Leishmania* is not inhibited by myoglobin. Binding of hemoglobin was also not competed by hemocyanin (protein containing copper-tetrapyrrole) or transferrin (Fig. 2).

At higher temperatures (25 °C or 37 °C), hemoglobin is internalized and degraded up to the moniodotyrosine level (Fig. 3, a and b). Uptake of hemoglobin at 37 °C reached a steady state within 60 min, while the degradation continued to show a linear rise, indicating simultaneous uptake and degradation of hemoglobin by the parasite (Fig. 4). Hemin and globin did not show any significant competition for uptake (Fig. 5a). Globin also did not compete for degradation of hemoglobin, but hemin caused substantial inhibition of hemoglobin degradation (Fig. 5b). It is possible that hemin-mediated impairment of hemoglobin degradation in *Leishmania* might act as a feedback mechanism precluding intracellular accumulation of excessive heme.

Three lines of evidence suggest that after initial binding with cell surface receptor hemoglobin is internalized by *Leishmania*. 1) Electron microscopic studies showed that at 4 °C hemoglobin bound to specific sites located in the flagellar pocket of *Leishmania* (Fig. 6, b and e), whereas at 37 °C the hemoglobin was found distributed throughout the cell. After initial binding with

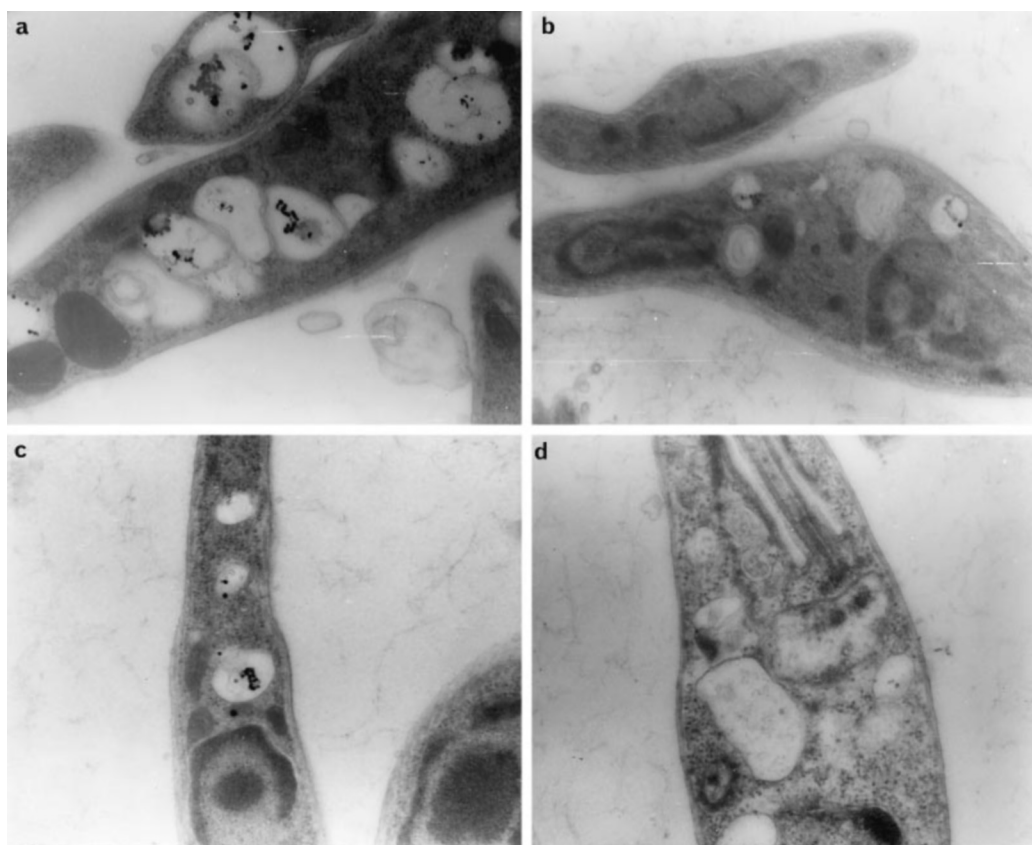


FIG. 7. Electron micrograph showing the intracellular localization of the hemoglobin-gold inside the *Leishmania*. Promastigotes (5×10^5) were incubated with hemoglobin-gold ($1 \mu\text{g/ml}$) for 1 h in the absence (a and c) or presence (b and d) of excess unlabeled hemoglobin at 37°C (a and b) or 25°C (c and d). The cells were washed and processed for electron microscopy. Original magnification, $\times 25,000$.

TABLE I
Quantitation of hemoglobin-gold uptake by *L. donovani*

Addition	Temperature °C	No. of gold particles ^a
Hb-gold	37	371
Hb-gold + Hb	37	86
Hb-gold	25	124
Hb-gold + Hb	25	31
BSA-gold	37	38

^a Number of gold particles present in 50 *Leishmania*.

the sites localized in the flagellar pocket at 4°C , hemoglobin was rapidly internalized by the cells as shown by the fluorescence microscopic studies (Fig. 8). When the cells were warmed for only 2 min, most of the hemoglobin was detected inside the cell presumably in the early endosome-like compartments (Fig. 8b). After 5 min, hemoglobin was detected in discrete intracellular vesicles (Fig. 8c) and was eventually dispersed throughout the cell body by 15 min (Fig. 8d). 2) When the cells were incubated with hemoglobin-gold at 37°C or 25°C for 1 h, gold particles were found to be localized in discrete intracellular vesicles throughout the cell body (Fig. 7, a and c). Competition of hemoglobin-gold uptake by free hemoglobin indicated the specificity of hemoglobin endocytosis (Fig. 7, b and d; Table I). 3) Biotinylated hemoglobin bound by *Leishmania* at 4°C could be detected in endocytic vesicles isolated after short incubation of the cells at 37°C (data not shown). These results suggest that, after high affinity binding with specific sites on the cell surface, hemoglobin is internalized through a vesicular transport pathway.

In contrast to mammalian cells, endocytosis occurs in the trypanosomatid parasites through the flagellar pocket, although the exact nature of these compartments is not known

(4). It has been shown that, after binding to specific receptors localized in the flagellar pocket, rapid endocytosis of macromolecular nutrients such as low density lipoprotein or transferrin occurs in trypanosomatid parasites. We have demonstrated that the hemoglobin-binding sites in *Leishmania* located in the flagellar pocket mediate rapid endocytosis of bound hemoglobin. Transferrin receptor is known to be recycled in mammalian cells (31). In contrast, it has been demonstrated in *Trypanosoma* that both transferrin receptor along with the ligand are targeted to a lysosome-like compartment (32). Our data showing degradation of ^{125}I -hemoglobin to trichloroacetic acid-soluble material primarily to the level of monoiodotyrosines are also consistent with existence of such a degradative compartment in *Leishmania*. The insensitivity of hemoglobin degradation by *Leishmania* to potent inhibitors of lysosomal degradative processes in mammalian cells chloroquine, monensin, or ammonium chloride (33, 34) remains to be explained, but could presumably be related to the relatively higher pH values (≥ 6) of endolysosomal compartments in protozoan parasites (35). It will be interesting to determine the nature of the intracellular compartments to establish the pathway of hemoglobin transport inside the cell. It would also be interesting to determine whether the intracellular transport of hemoglobin is regulated by vesicle fusion. Actually, in some of the micrographs, we were able to detect dumbbell-shaped vesicles containing gold particles, indicating the possible fusion of different intracellular compartments (Fig. 7a). However, further work would be needed to establish the nature of the compartments and the mechanism of the fusion.

A single biotinylated protein of 46 kDa was revealed when solubilized membranes from surface biotinylated intact *Leishmania* adsorbed by hemoglobin-agarose beads were subjected to SDS-PAGE and Western blotting with avidin-HRP (Fig. 9).

FIG. 8. Time course of internalization at 37 °C of hemoglobin bound at 4 °C by *Leishmania* promastigotes. Cells (5×10^7) were incubated at 4 °C for 2 h with hemoglobin (5 μ g/ml) to allow binding with cell surface. Subsequently, cells were washed to remove unbound hemoglobin and incubated at 37 °C for 0 min (a), 2 min (b), 5 min (c), and 15 min (d). Hemoglobin molecules were visualized by anti-hemoglobin antibody probed with respective anti-mouse FITC-labeled second antibody. There was no staining in cells incubated in absence of hemoglobin or the cells processed without anti-hemoglobin antibodies.

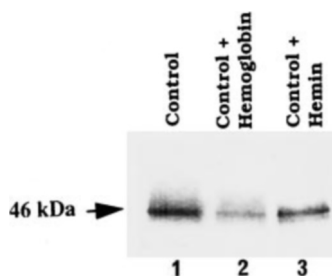
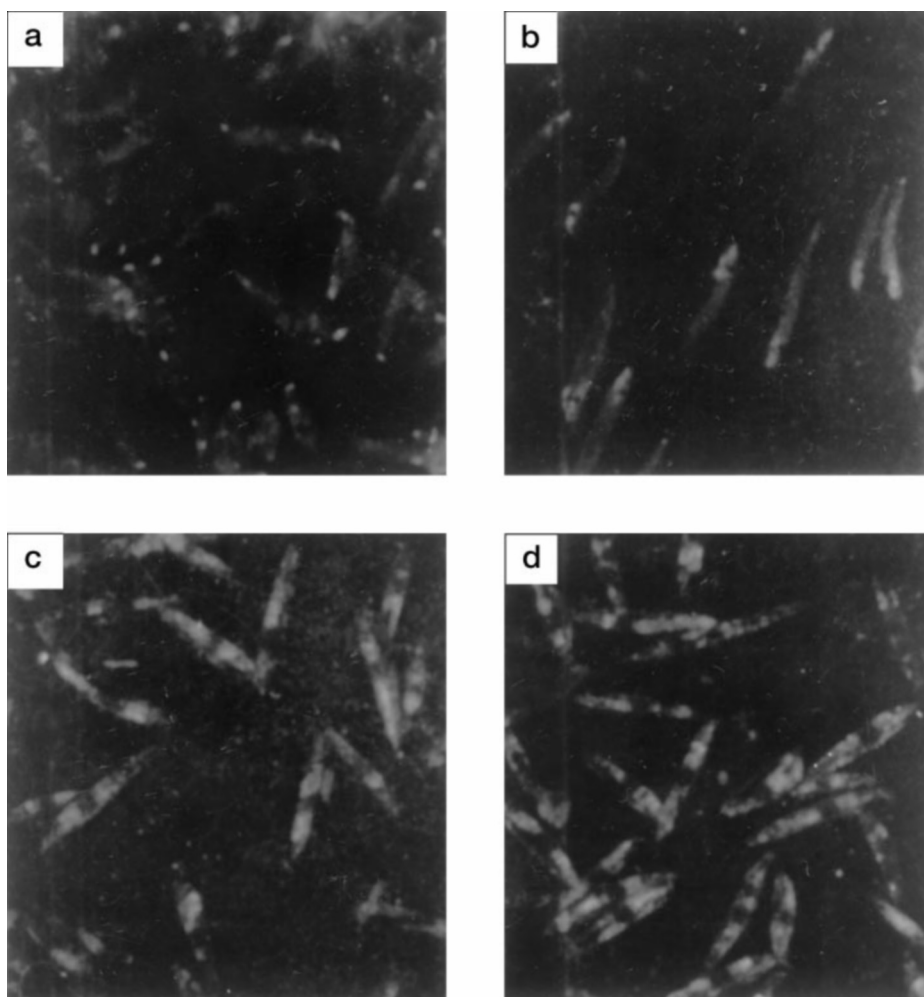


FIG. 9. Western blot analysis of proteins recovered by hemoglobin-agarose affinity purification of detergent extracts of *L. donovani* membranes. Promastigotes were biotinylated, and the membrane fraction obtained thereafter was solubilized and incubated with hemoglobin-agarose beads as described under "Experimental Procedures." The proteins eluted from hemoglobin-agarose beads were subjected to SDS-PAGE, transferred to nitrocellulose membrane, and stained with avidin-HRP. Lane 1, control. Lanes 2 and 3, membrane fractions pre-incubated with hemoglobin and hemin, respectively.

This further confirms that the hemoglobin-binding protein is present on the *Leishmania* membrane, as only cell surface proteins would be biotinylated under the conditions employed (29). Furthermore, preincubation of the membrane fraction with hemoglobin could compete this band out, suggesting the hemoglobin-binding specificity of the 46-kDa protein. Preincubation with hemin did not result in any competition, indicating that the hemoglobin-binding protein on the *Leishmania* membrane does not interact with hemin.

Thus, in the present investigation, we have shown that hemoglobin binds with high affinity through specific binding sites located in the flagellar pocket of *Leishmania*. The binding of

hemoglobin with *Leishmania* surface membrane is mediated through a putative receptor protein of apparent molecular mass of 46 kDa. After high affinity binding with cell surface receptor, hemoglobin is rapidly internalized into discrete intracellular compartments. To the best of our knowledge, this is the first report demonstrating the existence of a vesicular pathway for transport of hemoglobin in *Leishmania*. It will be interesting to determine the mechanism of hemoglobin trafficking inside the *Leishmania* and the role of Rab-like GTPases in this transport. Further studies are in progress to determine the role of signal transduction intermediates in the mechanism of hemoglobin transport.

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