A Nonpermeant Biotin Derivative Gains Access to the Parasitophorous Vacuole in Plasmodium falciparum-infected Erythrocytes Permeabilized with Streptolysin O*

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In its host erythrocyte, the malaria parasite Plasmodium falciparum resides within a parasitophorous vacuole, the membrane of which forms a barrier between the host cell cytosol and the parasite surface. The vacuole is a unique compartment because it contains specific proteins that are believed to be involved in cell biological functions essential for parasite survival. As a prerequisite for the characterization of the vacuolar proteome, we have developed an experimental approach that allows the selective biotinylation of soluble vacuolar proteins. This approach utilizes nonpermeant biotin derivatives that can be introduced into infected erythrocytes after selective permeabilization of the erythrocyte membrane with the pore-forming protein streptolysin O. The derivatives gain access to the vacuolar lumen but not to the parasite cytosol, thus providing supportive evidence for the existence of nonselective pores within the vacuolar membrane that have been postulated based on electrophysiological studies. Soluble vacuolar proteins that are biotin-labeled can be isolated by affinity chromatography using streptavidin-agarose.

Plasmodium falciparum, the causative agent of the most severe form of malaria, invades human erythrocytes, where this unicellular parasite develops within the so-called parasitophorous vacuole (PV). The parasitophorous vacuolar membrane (PVM) is formed during the process of invasion, and it contains lipids from the erythrocyte plasma membrane (1, 2) and, presumably, parasite-derived proteins and lipids (3). This membrane presents a barrier between parasite and host cell cytosol, although electrophysiological studies in Plasmodium-infected erythrocytes suggest the existence of nonselective pores within the PVM that allow passive bidirectional diffusion of small molecules (4, 5). With respect to biogenesis and protein contents, the PV differs significantly from endocytic vacuoles (6), with the most apparent difference being the almost cytosolic pH of the vacuole (7, 8). In recent years, many observations have indicated a number of cell biologically relevant processes within the vacuole that are important for parasite survival.

In the course of the infection, several parasite proteins are exported into the erythrocyte, and some of these proteins, such as the members of the PEPTEM1 family, are key molecules involved in the pathogenesis of malaria (9). For at least some exported proteins, the PV is a transit compartment from which they are transported into the host cell cytosol (10–12). Translocation across the vacuolar membrane must be a selective process because other proteins are retained within the PV (13, 14). Recently, it has been suggested that proteins destined for the parasite apicoplast, a unique plastid-like intracellular organelle of apicomplexan parasites, also may be trafficked via the PV (15). Thus, the PV must contain a machinery involved in protein sorting. Upon completion of parasite development and multiplication, merozoites, the invasive stages, are released from the infected cell. Release of merozoites from the PV can be prevented by treatment of infected erythrocytes (IRBCs) with protein synthesis inhibitors (16). In addition, evasion of merozoites is accompanied by a specific sequential cleavage of the major merozoite surface protein 1 that is essential for the subsequent reinvasion of noninfected cells (17). These data suggest a role of vacuolar proteases in the release of parasites from the infected host cell. Collectively, the current observations underscore the notion that the PV is a unique intracellular compartment critical for parasite survival within the host cell and that it most likely contains novel proteins of elementary functions.

The volume of the vacuole compared with the volumes of the parasite cytosol and the erythrocyte cytosol, respectively, is very small. Estimates based on morphological data suggest a 1:10,000 ratio of vacuolar volume to erythrocyte cytosol in IRBCs infected with stages of the parasite that have completed ~30 h of the 48-h intraerythrocytic development (12). Cell fractionation experiments on IRBCs infected with the same developmental stages show that more than 70% of the total protein are erythrocyte cytosolic proteins, predominantly hemoglobin (18). Therefore, the isolation of vacuolar proteins is difficult; consequently, only a few vacuolar proteins have been identified thus far. In this report, we describe a novel strategy for the identification of vacuolar proteins as a prerequisite for a comprehensive proteome analysis of this compartment.
Biotin derivatives sulfosuccinimidyl-2-biotinyl ether and sulfosuccinimidyl-6-biotinyl hexahydrolactone (Sigma) were dissolved in 20 mM Tris-HCl, pH 7.4, and ASB (0.05% sodium azide, 0.1% NaN3) before biotinylation.

For the biotinylation of intact erythrocytes, 10⁹ parasitized or nonparasitized erythrocytes were washed three times in PBS containing 0.6 mM CaCl₂ and 1 mM MgCl₂, pH 7.6 (PBS⁺), and then incubated in PBS⁺ containing 1 mg ml⁻¹ sulfosuccinimidyl-NHS-biotin for 30 min on ice. Cells were sedimented by centrifugation at 10,000 × g for 15 s at 4 °C. The supernatant was analyzed photometrically at 412 nm for the detection of radiolabeled proteins. For two-dimensional PAGE, soluble parasite and vacuolar proteins were transferred to 12% slab gels and separated in the second dimension by isoelectric focusing.

When proteins were labeled with the cleavable biotin derivative sulfo-NHS-biotin, separation was on 5–20% gradient gels under nonreducing conditions. Protein samples were visualized by silver staining before exposure to x-ray film for the detection of radiolabeled proteins. For indirect immunofluorescence, the individual strips were consecutively incubated in equilibration solution A and B (50 mM Tris-HCl, pH 6.8, 8 mM urea, 30% (v/v) glycerol, 2% (w/v) SDS, complemented with 3.5 mg ml⁻¹ DTT (solution A) or 45 mg ml⁻¹ iodoacetamide instead of DTT (solution B)), each for 15 min. The equilibrated strips were transferred to 12% slab gels and separated in the second dimension. After drying of the gels, radiolabeled bands were visualized by autoradiography.

**Indirect Immunofluorescence Assay**—Permeabilized and biotin-treated IRBCs were spread on glass slides and air-dried. Subsequently, they were fixed in acetone-methanol (1:1) for 10 min at 20 °C. The slides were incubated with the primary antibodies for 2 h at room temperature. The monoclonal anti-band 3 IgG was diluted 1:20, and the polyclonal rabbit antisera used for SERP and aldolase antibodies, were diluted 1:10 in PBS, pH 7.2. After three washes with PBS, pH 7.2, slides were probed with a mixture of the corresponding secondary antibody (1:100) and Cy3-conjugated streptavidin (1:200) for 30 min at room temperature in the dark. Autofluorescence and nonspecific fluorescence levels were determined by viewing control samples of either biotin-labeled membranes or treated membranes using Cy3-conjugated streptavidin and secondary antibodies. The cells were mounted in glycerol containing 0.1% of the antifade reagent 1,4-diazobicyclo(2,2,2)-octane. Fluorescence microscopy was performed using a Leica TCS SP2 laser scanning confocal microscope.
internalization of biotin across the intact host cell plasma membrane is limited and that the excess of erythrocyte cytosolic proteins prevented efficient labeling of vacuolar proteins.

**Biotin Accumulates in the Parasitophorous Vacuole in Permeabilized Infected Erythrocytes**—Although the vacuolar membrane forms a barrier between the parasite surface and the host cell cytosol, it is most conceivable that the PVM allows the transport of nutrients essential for parasite survival. Using a patch-clamp technique, Desai et al. (4) measured high conductance channels that are permeable to organic and inorganic anions and cations. The channels are open 98% of the time, and they are highly abundant. In bilayers, this channel allows passage of molecules up to 1,400 Da (5), which is similar to the nonselective pores discovered in the PVM of the taxonomically related parasite Toxoplasma (25). The existence of these pores in Plasmodium-infected erythrocytes has not been verified biochemically, but if they exist, they should allow access of membrane-impermeable biotin derivatives that are ~600 Da in size.

To increase access of biotin to the interior of the infected erythrocyte, we utilized SLO to permeabilize the erythrocyte plasma membrane. In an initial assessment of our experimental strategy, infected erythrocytes were permeabilized and, after complete removal of the erythrocyte cytosol, incubated with the membrane-impermeable sulfo-NHS-LC-biotin, which was detected using a fluorescent streptavidin derivative (Fig. 2). The images show a distinct ring around the periphery of the intracellular parasite. The biotin co-localizes with the vacuolar marker protein SERP (Fig. 2A). This location is distinct from the location of the erythrocyte membrane protein band 3 and from the location of the parasite cytosolic protein aldolase (Fig. 2B and C). In some parts, the streptavidin and the antibody to band 3 co-localize; this is due to the fact that proteins of the erythrocyte plasma membrane also react with the biotin. Little if any biotin was detectable in the erythrocyte cytosol and in the parasite cytosol, respectively. A ring-like staining around the periphery of the parasite can be attributable to a reaction of the biotin with membrane proteins of the PVM and with soluble vacuolar proteins. Because the resolution of fluorescence microscopy does not allow discrimination between a staining of the PVM and a luminal staining, a more detailed biochemical analysis was performed to assess the biotinylation of soluble vacuolar proteins.

**Biotin-labeled Parasite Proteins Bind to SAv-agarose**—Because mammalian erythrocytes have lost the ability to synthesize proteins de novo, in infected erythrocytes, radiolabeled amino acids are incorporated exclusively into parasite proteins. The following experiment was designed to optimize the biotinylation conditions and the selective isolation of biotinylated proteins by SAv-agarose. Infected cells were cultivated for 30 min in the presence of L-[^35S]methionine, and subsequently, the erythrocyte plasma membrane was permeabilized with SLO to release the soluble contents of the erythrocyte cytosol. In all experiments, the integrity of the PVM and the parasite plasma membrane was assessed as described previously (10). Parasite-containing vacuoles were lysed by repeated freezing and thawing, and the fraction of soluble proteins was collected after centrifugation. One aliquot of the lysate was treated with SAv-agarose and another aliquot remained untreated. Both samples were incubated with SAv-agarose beads, and bound proteins were eluted by boiling the beads in SDS sample buffer. The fractions of unbound and bound proteins were analyzed by SDS-PAGE and autoradiography (Fig. 3A). No radiolabeled proteins from the nonbiotinylated sample bound to SAv-agarose (Fig. 3A, lane 2), whereas after biotinylation, most radiolabeled proteins were found in the fraction of bound proteins (Fig. 3A, lane 4). It is noteworthy that the pattern of

**Fig. 1.** Infected erythrocytes are permeable for a nonpermanent biotin derivative. Noninfected (RBC) and infected (iRBC) erythrocytes were incubated with sulfo-NHS-SS-biotin, and a sample from each reaction was subsequently treated with trypsin (lanes 3, 4, 7, and 8) to cleave externally exposed proteins. Cells were lysed and separated into a fraction of soluble proteins (lanes 1, 3, 5, and 7) and into a membrane fraction (lanes 2, 4, 6, and 8). Proteins, corresponding to 2 × 10⁷ cells, were electrophoresed through a 5–15% nonreducing SDS-polyacrylamide gel, transferred to a nitrocellulose filter, and probed with alkaline phosphatase-conjugated SAv.

**RESULTS AND DISCUSSION**

A Membrane-impermeable Biotin Derivative Gains Access into Intact Infected Erythrocytes—The intracellular parasite depends on the acquisition of nutrients from the extracellular milieu and from the host cell cytosol. Extracellular nutrients must be taken up across three membranes, the erythrocyte plasma membrane, the PVM, and the parasite plasma membrane. In the course of infection, the erythrocyte plasma membrane undergoes major alterations. Most notably, the transport properties of the membrane change, increasing the influx rates for a number of low molecular weight solutes. The identification of the proteins that mediate these novel transport properties is being pursued intensely (for current reviews, see Refs. 22–24). In preliminary experiments designed to study transport of small solutes across the erythrocyte membrane, we noticed that the membrane-impermeable sulfo-NHS-SS-biotin gained access to the interior of erythrocytes infected with trophozoite-stage parasites, but not to noninfected erythrocytes. Intact noninfected and infected erythrocytes were incubated with sulfo-NHS-SS-biotin. Cells were lysed in water by repeated freezing and thawing, and soluble proteins were separated from the membrane fraction by centrifugation. Whereas in noninfected cells, no biotin labeling of soluble proteins was detectable (Fig. 1, lanes 1 and 3), soluble proteins were found to be biotin-labeled in infected cells (Fig. 1, lanes 5 and 7). When intact erythrocytes were treated with trypsin after the biotin reaction, the pattern of biotin-labeled proteins in the membrane fraction changed, due to proteolytic cleavage of externally exposed protein domains (Fig. 1, lanes 2, 4, 6, and 8). More importantly, trypsin treatment had little effect on the pattern of biotin-labeled soluble proteins in infected cells (Fig. 1, lanes 5 and 7). This result demonstrates that the protease, unlike the biotin derivative, was excluded from the erythrocyte cytosol. Most likely, access of biotin into intact erythrocytes is due to the novel transport properties of the erythrocyte membrane, and we are currently investigating the pathways involved in this process. We never observed that biotin gained access to vacuolar proteins in infected erythrocytes using these experimental conditions (data not shown). We anticipate that
biotinylated proteins differed from that of the nonbiotinylated proteins and that the bands appeared less focused. This observation is attributable to the variant degrees of biotin labeling of individual polypeptide chains. To assess the effects of biotin incorporation on the electrophoretic motility of proteins, BSA was incubated with different molar ratios of sulfo-NHS-LC-biotin and analyzed by SDS-PAGE. As shown in Fig. 3, the electrophoretic motility of BSA is altered depending on the degree of biotinylation.

The subsequent experiments were devised to demonstrate compartment-specific biotinylation of soluble parasite proteins in permeabilized IRBCs. The so-called GBP, the SERP, and parasite aldolase (PfALD) are soluble marker proteins that are located in different compartments of the infected erythrocyte. Aldolase is restricted to the parasite cytosol (19), SERP is restricted to the vacuole (13), and GBP is found both inside the vacuole and within the erythrocyte cytosol (10). In the first step, it was confirmed that these proteins can be biotinylated and that they bind to SAv-agarose, provided that they are accessible to biotin. This was assessed using lysates of permeabilized infected erythrocytes; infected erythrocytes were permeabilized with SLO, and after complete removal of the erythrocyte cytosol, a lysate containing soluble proteins of the parasite cytosol and of the PV was prepared. One aliquot of soluble proteins was treated with sulfo-NHS-LC-biotin as described above. Another aliquot remained untreated. The samples were incubated with SAv-agarose beads, and the bound and unbound proteins were analyzed separately by immunoblotting using antisera to the respective marker proteins (Fig. 4A). In the sample of nonlabeled proteins, the three marker proteins were recovered in the fraction of unbound proteins (Fig. 4A, compare lanes 1 and 2). After biotin treatment, GBP, SERP, and PfALD were recovered in the fraction of bound proteins (Fig. 4A, compare lanes 3 and 4). In the case of GBP and SERP, recovery was almost quantitative. Although recovery was not entirely complete in the case of PfALD, these results demonstrate that the marker proteins are biotinylated and can be isolated on SAv-agarose.

After confirmation that all marker proteins are biotinylatable, IRBCs were permeabilized with SLO, washed to remove biotinylated proteins differed from that of the nonbiotinylated proteins and that the bands appeared less focused. This observation is attributable to the variant degrees of biotin labeling of individual polypeptide chains. To assess the effects of biotin incorporation on the electrophoretic motility of proteins, BSA was incubated with different molar ratios of sulfo-NHS-LC-biotin and analyzed by SDS-PAGE. As shown in Fig. 3, the electrophoretic motility of BSA is altered depending on the degree of biotinylation.

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the contents of the erythrocyte cytosol, and incubated with sulfo-NHS-LC-biotin. Upon removal of free biotin and lysis of parasites, soluble proteins were immunoprecipitated using antibodies to SERP and GBP, respectively. Precipitated proteins were separated by SDS-PAGE and transferred to nitrocellulose filters. Initially, the filters were probed with the antisera against GBP and SERP, respectively, separated by SDS-PAGE, and transferred to nitrocellulose filters (lanes 1 and 3). The same filters were re-probed with peroxidase-conjugated SAv, which was subsequently detected by a chemiluminescence reaction (lanes 2 and 4). The incorporation of biotin into aldolase was analyzed in the same way (lanes 5 and 6), except that soluble proteins were separated electrophoretically without prior immunoprecipitation. C, the lysate was reacted with SAv-agarose beads, and the fractions of bound (lanes 1 and 3) and unbound (lanes 2 and 4) proteins were analyzed as described in B for the incorporation of biotin (lanes 1 and 2) and the presence of the respective marker proteins (lanes 3 and 4).
with sulfo-NHS-LC-biotin. After cell lysis, soluble proteins cytes, one gel was silver-stained (Fig. 6 A, lanes 1 and 2), untreated sample; lanes 3 and 4, biotinylated proteins. B, PfBip was immunoprecipitated from a sample of biotin-treated cells, and the precipitate was analyzed by immunoblotting using either the specific antiserum (lane 1) or peroxidase-conjugated SAv (lane 2). C, biotinylated proteins were bound to SAv beads, and the beads were washed with either HEPES buffer (lanes 1 and 2) or HEPES buffer containing 5 mM ATP (lanes 3 and 4). Proteins that remained bound to the beads (lanes 2 and 4) and unbound proteins (lanes 1 and 3) were analyzed for the presence of SERP and PfBip.

but it had no effect on the isolation of SERP (Fig. 5C) or of other biotin-labeled proteins.

Two-dimensional Gel Electrophoretic Analysis of Biotin-labeled Parasite Proteins—As a prerequisite for the identification of novel vacuolar proteins, IRBCs were labeled metabolically with L-[35S]methionine, permeabilized with SLO, and treated with sulfo-NHS-LC-biotin. After cell lysis, soluble proteins were separated in triplicate on different two-dimensional gels using a pH gradient of 4–7 in the first dimension. To estimate the relative abundance of proteins in permeabilized erythrocytes, one gel was silver-stained (Fig. 6A), and one gel was stained with Coomassie Blue (Fig. 6B). As expected, silver staining revealed the most complex protein pattern. The most abundant proteins were also detectable using the less sensitive Coomassie Blue stain. To compare biotinylated proteins versus total parasite proteins, proteins of a third gel were transferred to a nitrocellulose filter. The filter was exposed to x-ray film to analyze the pattern of radiolabeled proteins (Fig. 6C). Subsequently, biotin-labeled proteins were detected using alkaline phosphatase-conjugated SAv (Fig. 6D). A comparison of the biotin-labeled proteins and the radiolabeled proteins clearly demonstrates that the pattern of biotin-labeled proteins is less complex and that it does not simply reflect the pattern of the most abundant radiolabeled proteins. In contrast, the most intensely radiolabeled proteins appear not to be biotin-labeled. Considering that cytosolic housekeeping proteins of the parasite, such as enzymes of the glycolytic pathway, are likely to be highly abundant, this result underscores the notion that the incorporation of the biotin is selective. In fact, when a matrix-assisted laser desorption ionization time-of-flight analysis was carried out using the two most prominent spots (I and II), they were identified unequivocally as P. falciparum enolase and as P. falciparum ornithine aminotransferase, respectively (data not shown). Biotin-labeled proteins form characteristic strings of individual spots. We attribute this pattern to various degrees of biotin incorporation into the same polypeptide chain. Because each sulfo-NHS-LC-biotin adds a negative charge, molecules with a high incorporation of the derivative are expected to migrate toward the acidic end of the gradient. The shift to the acidic end of the gradient also correlates with a slight increase in molecular size. A detailed comparison reveals 12 prominent spots, circled in Fig. 6, A–D, which represent highly abundant parasite proteins that are detectable by Coomassie Blue staining. Nine of these spots appear negatively stained in Fig. 6D because of the high local concentration of nonbiotinylated protein. Thirty-nine protein spots were identified that were both biotinylated and radiolabeled and that therefore fulfill the criteria expected for vacuolar proteins. Some of these spots may represent identical polypeptides at various degrees of biotinylation are marked with circles. Proteins that are both biotinylated and radiolabeled are indicated by Arabic numerals; spots that may represent identical polypeptides at various degrees of biotinylation are marked with lowercase letters. I, P. falciparum enolase; II, P. falciparum ornithine aminotransferase.

Fig. 5. Co-isolation of PfBip with biotinylated proteins. A, infected erythrocytes were permeabilized, and one aliquot of the cells was treated with sulfo-NHS-LC-biotin. Lysates of cells were prepared and incubated with SAv-agarose beads. The fraction of unbound proteins (lanes 1 and 3) and the fraction of bound proteins (lanes 2 and 4) were analyzed for the presence of PfBip using a specific antiserum. Lanes 1 and 2, untreated sample; lanes 3 and 4, biotinylated proteins. B, PfBip was immunoprecipitated from a sample of biotin-treated cells, and the precipitate was analyzed by immunoblotting using either the specific antiserum (lane 1) or peroxidase-conjugated SAv (lane 2). C, biotinylated proteins were bound to SAv beads, and the beads were washed with either HEPES buffer (lanes 1 and 2) or HEPES buffer containing 5 mM ATP (lanes 3 and 4). Proteins that remained bound to the beads (lanes 2 and 4) and unbound proteins (lanes 1 and 3) were analyzed for the presence of SERP and PfBip.

Fig. 6. Protein pattern of putative vacuolar proteins. Proteins in intact infected erythrocytes were metabolically labeled, and cells were permeabilized and treated with biotin. The fraction of soluble proteins, each corresponding to 2 × 10⁵ infected erythrocytes, was subjected to two-dimensional gel electrophoresis using a pH gradient of 4–7 in the first dimension and 12% SDS-PAGE in the second dimension. A, silver-stained gel. B, Coomassie Blue-stained gel. C and D, proteins from a third two-dimensional gel were transferred to a nitrocellulose filter. The filter was first exposed to x-ray film to visualize metabolically labeled parasite proteins (C) and subsequently reacted with alkaline phosphatase-conjugated SAv to visualize biotinylated proteins (D). The most prominent spots that were identified as parasite proteins are marked with circles. Proteins that are both biotinylated and radiolabeled are indicated by Arabic numerals; spots that may represent identical polypeptides at various degrees of biotinylation are marked with lowercase letters. I, P. falciparum enolase; II, P. falciparum ornithine aminotransferase.

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