Transport of the essential nutrient isoleucine in human erythrocytes infected with the malaria parasite \textit{Plasmodium falciparum}

Rowena E. Martin\textsuperscript{1} and Kiaran Kirk\textsuperscript{1}

\textsuperscript{1}School of Biochemistry and Molecular Biology, The Australian National University, Canberra, Australia

The intraerythrocytic malaria parasite derives much of its requirement for amino acids from the digestion of the hemoglobin of its host cell. However, one amino acid, isoleucine, is absent from adult human hemoglobin and must therefore be obtained from the extracellular medium. In this study we have characterized the mechanisms involved in the uptake of isoleucine by the intraerythrocytic parasite. Under physiologic conditions the rate of transport of isoleucine into human erythrocytes infected with mature trophozoite-stage \textit{Plasmodium falciparum} parasites is increased to approximately 5-fold that in uninfected cells, with the increased flux being via the new permeability pathways (NPPs) induced by the parasite in the host cell membrane. Transport via the NPPs ensures that protein synthesis is not rate limited by the flux of isoleucine across the erythrocyte membrane. On entering the infected erythrocyte, isoleucine is taken up into the parasite via a saturable, ATP-, Na\textsuperscript{+}, and H\textsuperscript{+}-independent system which has the capacity to mediate the influx of isoleucine in exchange for leucine (liberated from hemoglobin). The accumulation of radiolabeled isoleucine within the parasite is mediated by a second (high-affinity, ATP-dependent) mechanism, perhaps involving metabolism and/or the concentration of isoleucine within an intracellular organelle. (Blood. 2007;109:2217-2224)

© 2007 by The American Society of Hematology

\textbf{Introduction}

The growth and multiplication of the intraerythrocytic malaria parasite is dependent on the presence in the extracellular solution of isoleucine,\textsuperscript{1-4} an amino acid that is absent from adult human hemoglobin\textsuperscript{5,6} and which the parasite is therefore unable to obtain from this source. Normal human erythrocytes take up isoleucine via a saturable carrier, the L system.\textsuperscript{7-10} In an early study McCormick\textsuperscript{11} reported that the rate of isoleucine uptake into \textit{Plasmodium knowlesi}-infected monkey erythrocytes was increased relative to that in uninfected cells, and that isoleucine was accumulated up to 20 times more than in normal cells. However, the metabolism of isoleucine was not assessed, and the increased uptake of isoleucine in infected erythrocytes may have been due, at least in part, to the high rate of incorporation of isoleucine into protein. Sherman and Tanigoshi\textsuperscript{12} observed that infection by \textit{Plasmodium lophurae} enhanced the permeability of duck erythrocytes to amino acids and concluded that the transport of most amino acids across the parasite plasma membrane occurs by simple diffusion, rather than via a carrier-mediated process. However, the transport of isoleucine was not investigated. In later studies, Ginsburg et al\textsuperscript{13} and Kirk et al\textsuperscript{14} reported there to be an increase in the permeability of \textit{Plasmodium falciparum}-infected human erythrocytes to a range of amino acids, including isoleucine.\textsuperscript{13} By contrast, Elford et al\textsuperscript{15} reported the rate of isoleucine transport into human erythrocytes to be unaffected by \textit{P falciparum} infection.

In this study we have investigated the transport of isoleucine across the infected erythrocyte membrane, demonstrating a key role for the new permeability pathways induced by the parasite in the host erythrocyte membrane. Following on from this we have characterized the mechanism by which this essential nutrient, having entered the infected erythrocyte, is taken up into the intracellular parasite itself.

\textbf{Materials and methods}

\textbf{Cell culture, cell preparations, and solutions}

Human erythrocytes (type O\textsuperscript{+}) infected with \textit{P falciparum} (strain FAF6) were cultured and synchronized as described elsewhere.\textsuperscript{16} Experiments were carried out using trophozoite-infected cells (approximately 30-35 hours after invasion).

\textit{P falciparum}-infected erythrocytes were concentrated to between 70\% and 98\% parasitemia by Percoll centrifugation,\textsuperscript{17} then washed (2 \times 1) in the appropriate solution prior to experimentation. Parasites were isolated from their host erythrocytes by saponin-permeabilization of the erythrocyte and parasitophorous vacuole membranes, as described elsewhere.\textsuperscript{18}

A number of different solutions were used in this study. Their composition was as follows: solution A [130 mM NaCl, 25 mM HEPES, 5 mM KCl, 20 mM glucose, 0.2 mM hypoxanthine, 25 mg/L gentamycin sulfate, supplemented with RPMI 1640 vitamins and glutathione (Invitrogen, Carlsbad, CA) 1 \times; pH 7.4/7.1]; solution B [135 mM NaCl, 25 mM HEPES, 5 mM KCl, 20 mM glucose, 1 mM MgCl\textsubscript{2}; pH 7.1]; solution C (glucose free; same as solution B but without glucose); solution D (Na\textsuperscript{+} free; same as solution B but containing choline-Cl in place of NaCl); solution E (120 mM NaCl, 25 mM HEPES, 25 mM MES, 5 mM KCl, 20 mM glucose, 1 mM MgCl\textsubscript{2}; pH 7.3/5.5).

In experiments carried out under physiologic conditions, at 37°C, the extracellular solution was supplemented with amino acids at the following concentrations (each falling within the normal physiologic range\textsuperscript{19}): alanine (356 \text{M}), arginine (88 \text{M}), asparagine (13 \text{M}), aspartate (13 \text{M}), cystine (37 \text{M}), glutamate (57 \text{M}), glutamine (476 \text{M}), glycine (217 \text{M}), histidine (70 \text{M}), isoleucine (0.7 \text{M}), leucine (39 \text{M}), lysine (10 \text{M}), methionine (3.5 \text{M}), phenylalanine (50 \text{M}), proline (20 \text{M}), serine (5.8 \text{M}), threonine (35 \text{M}), tryptophan (1.5 \text{M}), tyrosine (50 \text{M}), valine (31 \text{M}).
μM), histidine (85 μM), hydroxyproline (8 μM), isoleucine (70 μM), leucine (100 μM), lysine (163 μM), methionine (17 μM), phenylalanine (100 μM), proline (165 μM), serine (128 μM), threonine (112 μM), tryptophan (50 μM), tyrosine (62 μM), and valine (190 μM).

Radioisotope flux measurements

The influx of isoleucine into intact infected erythrocytes, uninfected erythrocytes, and isolated parasites was estimated from the uptake of [14C]-labeled isoleucine. Except where specified otherwise, influx was measured under nominally zero-trans conditions, achieved by depleting cells of amino acids by a series of washes and an incubation (~30 minutes, 37°C) in the appropriate amino acid–free saline prior to each experiment. The efflux of isoleucine and leucine from isolated parasites was measured using amino acid–depleted cells preloaded with radiolabeled amino acid.

In many experiments a combination of protein synthesis inhibitors was included in the medium. The inhibitors used were cycloheximide (40 μM), an inhibitor of eukaryote protein synthesis,22 and anisomycin (150 μM), an inhibitor of both eukaryote and prokaryote protein synthesis.23 Cycloheximide22 and anisomycin24 have been shown previously to inhibit protein synthesis in P falciparum–infected erythrocytes.25-27 Preliminary studies showed that it was found that a cycloheximide concentration of 20 μM inhibited protein synthesis by approximately 94%, and that the further addition of 75 μM anisomycin reduced protein synthesis to below detectable levels (data not shown).

Influx measurements in intact erythrocytes. Cells (70%-98% parasitemia) were suspended at 37°C in solution A, either with or without protein synthesis inhibitors. An appropriate volume of reaction medium (identical to the solution in which the cells were suspended but supplemented with all the common amino acids as well as [14C]isoleucine and, where appropriate, transport inhibitors) was dispensed into a microcentrifuge tube. Influx commenced with the addition of an equal volume of cell suspension, immediately followed by mixing. The final concentration of each amino acid in the extracellular medium (listed under “Cell culture, cell preparations, and solutions”) fell within the normal plasma range. At predetermined intervals (time courses) or after 15 seconds (fixed-time period experiments) aliquots of the suspension (typically 150 μL) were transferred to microcentrifuge tubes containing 300 μL dibutyl phthalate (density of 1.04 g/mL) layered over 25 μL 30% vol/vol perchloric acid. The tubes were centrifuged immediately (10 000g; 45 seconds) to sediment the cells through the oil and into the acid, thereby terminating the flux, lysing the cells, and precipitating the protein. The solution remaining on top of the oil was aspirated, together with the oil. Trichloroacetic acid (1 mL, 5% wt/vol) was added to the perchloric acid extract, and the sample was then centrifuged (10 000g; 2 minutes). The radioactivity in the supernatant solution (approximately 1 mL) was measured using a β-scintillation counter.

Measured transport rates were corrected to those for cells at 100% parasitemia by subtracting the contribution of the uninfected cells (determined on the basis of measurements on uninfected cells) then dividing by the fractional parasitemia.

Influx and efflux measurements in isolated parasites. Preliminary experiments (at 37°C) indicated that isoleucine transport across the parasite plasma membrane was extremely rapid; both influx and efflux assays were therefore performed at 20°C to slow the rate of transport. All such experiments were carried out in the presence of the protein synthesis inhibitors cycloheximide and anisomycin. The influx of [14C]isoleucine into isolated parasites was measured using a protocol similar to that used for intact erythrocytes. Except where specified otherwise, parasites were suspended in solution B, and uptake was measured in the absence of any unlabeled amino acids. Efflux experiments were performed using isolated parasites suspended in solution C and preloaded with either [14C]isoleucine or [14C]leucine in the presence of protein synthesis inhibitors. At time zero the cell suspension (1.08 mL) was mixed with 10 μL of either solution C or solution C containing 108 mM unlabeled leucine or isoleucine (yielding a final extracellular amino acid concentration of 1 mM). Isolated parasites were separated from the reaction medium by centrifugation through a blend of dibutyl phthalate and dioctyl phthalate (5:4; 1.015 g/mL) into 30% (vol/vol) perchloric acid. The acid extracts were processed for β-scintillation counting as described for the intact erythrocyte samples.

Estimation of extracellular space and intracellular water volume

The volume of extracellular solution trapped in infected cell pellets was determined using [14C]glutamate in conjunction with furosemide (200 μM), an effective inhibitor of glutamate uptake in parasitized erythrocytes.15-27 The extracellular volume in isolated parasite pellets was determined using [3H]aspartate (shown in preliminary experiments to be taken up very slowly relative to isoleucine) as an extracellular space marker. In both cases, the radiolabel was added to the cell suspension a few seconds before centrifugation.

Intracellular concentrations were calculated using previous estimates17 of the water volume of trophozoite-stage P falciparum–infected erythrocytes (75 fL) and saponin-isolated parasites (28 fL).

Protein synthesis measurements

Incorporation of [14C]isoleucine into protein was measured (at 37°C) both in intact infected erythrocytes and in isolated parasites. Cells were depleted of amino acids as described for the influx experiments and then resuspended in solution A. At time zero an aliquot of the cell suspension was mixed with an equal volume of solution A supplemented with all the common amino acids together with [14C]isoleucine and, where appropriate, transport inhibitors. Time courses or fixed time (5 minutes) incubations were carried out, and the samples were processed as described above. The acid-insoluble protein pellet was washed twice in 5% wt/vol trichloroacetic acid (1 mL), then water (1 mL), before being dissolved in 1 M NaOH (typically 0.1 mL). Bleach (25 mL of 12.5% wt/vol) was added to decolorize the solution, which was then diluted with water (1 mL) prior to β-scintillation counting.

Results

Human erythrocytes infected with P falciparum have increased permeability to isoleucine

Figure 1A shows time courses for the uptake of isoleucine (70 μM) into normal human erythrocytes and erythrocytes infected with the mature (trophozoite) form of P falciparum. In uninfected cells, the initial influx rate for isoleucine (estimated from the amount of isoleucine taken up over the first 15 seconds) under the conditions of the experiment was 110 ± 6 μmol/(1012 cells · hour) (mean ± SEM; n = 10), and the radiolabel equilibrated between the intracellular and extracellular solutions within 20 minutes, reaching a distribution ratio (ie, the intracellular concentration relative to that in the extracellular medium) of 0.91 ± 0.02 (n = 10). In parasitized cells under the same conditions the initial rate of influx was increased 5-fold, to 553 ± 27 μmol/(1012 cells · hour) (n = 10; P < .001, paired Student t test). The distribution ratio reached a value of 1 within 4 minutes and continued to accumulate thereafter, reaching a ratio of 1.48 ± 0.12 (again significantly higher than that in uninfected cells; P < .001) by 20 minutes.

Isoleucine is transported into intact parasitized erythrocytes via the endogenous L system and the NPPs

The transport of isoleucine by normal erythrocytes is predominately via the L system10 [Km = 10 ± 1 mM, Vmax = 9 ± 1 mmol/(1012 cells · hour) at 37°C; data not shown]. Figure 1B illustrates the effects on the influx of isoleucine into infected and uninfected erythrocytes of aminobicyclo[2.2.1]heptane-2-carboxylic acid (BCH, 20 mM), an inhibitor of the L system,28 and furosemide (200 μM), an effective inhibitor of the new permeability pathways (NPPs) induced by the parasite in the plasma membrane of infected erythrocytes.14

In uninfected erythrocytes, BCH reduced the influx of isoleucine by approximately 80%, consistent with the L system being the
major route of entry for isoleucine and mediating a flux of 91 ± 5 µmol/(10¹² cells · h) (n = 4). In infected cells the BCH-sensitive component of isoleucine influx into infected cells was 108 ± 13 µmol/(10¹² cells · h) (n = 4), similar to that measured in uninfected cells and therefore consistent with parasitization not having had a significant effect on the L system. In contrast to the situation in uninfected cells, the BCH-sensitive component of isoleucine influx accounted for only 20% of the total.

Furosemide had no effect on the transport of isoleucine into normal cells, but it reduced the influx of isoleucine into infected cells by approximately 80%.

**Furosemide slows protein synthesis in intact parasitized erythrocytes but not in isolated parasites**

Isoleucine is absent from adult human hemoglobin, and the parasite is dependent on the uptake of extracellular isoleucine to sustain protein synthesis. The rate of incorporation of exogenous isoleucine into protein therefore reflects the overall rate of protein synthesis by the intracellular parasite (NB the erythrocyte itself is incapable of protein synthesis and uninfected erythrocytes do not incorporate radiolabeled amino acids into acid-insoluble material; data not shown).

As illustrated in Figure 1C, the incorporation of isoleucine into protein by parasitized erythrocytes was approximately linear with time, occurring at a rate of 169 ± 6 µmol/(10¹² cells · h) (n = 10). This is significantly higher than the rate of transport of isoleucine into infected cells [ie, 110 ± 6 µmol/(10¹² cells · h); P < .001, paired t test], and significantly higher than the transport of isoleucine via the endogenous L system in parasitized cells [ie, 108 ± 13 µmol/(10¹² cells · h); P < .001, unpaired t test]. The capacity of the endogenous systems to transport isoleucine is therefore insufficient to meet the rate of incorporation of this amino acid into protein by the parasite.

Furosemide (200 µM) reduced the rate of incorporation of [¹⁴C]isoleucine into protein in intact parasitized cells by 60% [ie, from 169 ± 6 µmol/(10¹² cells · h) to 68 ± 4 µmol/(10¹² cells · h); n = 10; P < .001, paired t test]. The rate of incorporation of [¹⁴C]isoleucine into protein in isolated parasites [171 ± 4 µmol/(10¹² cells · h)] was the same as that in intact parasitized cells, consistent with the view that, in the absence of inhibitors, transport across the host cell membrane is not rate limiting for protein synthesis in the infected erythrocyte. The finding that in isolated parasites treated with furosemide the rate of protein synthesis [172 ± 5 µmol/(10¹² cells · h)] was the same as that in isolated parasites in the absence of furosemide shows that furosemide does not interfere with the mechanisms of protein synthesis. The reduction by furosemide of the protein synthesis rate in intact erythrocytes may therefore be attributed to an effect of furosemide on the erythrocyte plasma membrane.

Together, the data of Figure 1B and C are consistent with the supply of isoleucine via the NPP playing an essential role in the parasitized erythrocyte, ensuring that the transport of isoleucine across the erythrocyte membrane is not rate limiting for parasite protein synthesis.

**Uptake of isoleucine by isolated P falciparum trophozoites has 2 components**

The transport of isoleucine across the parasite plasma membrane was measured (at 20°C) in saponin-isolated P falciparum trophozoites. Figure 2 shows time courses for the uptake of isoleucine into isolated parasites suspended in a HEPES-buffered saline (solution B) containing approximately 0.08 µM isoleucine. Isoleucine was taken up rapidly by the isolated parasites, reaching a distribution ratio of close to 1, consistent with it having equilibrated between the intracellular and extracellular solutions, within 15 seconds. The initial rate of isoleucine influx, as estimated from the uptake over 15 seconds, was 525 ± 14 nmol/(10¹² cells · h); however, this represents an underestimate of the true influx rate as the 15-second incubation falls outside the initial linear phase of the uptake time course. Following the very rapid equilibration of the isoleucine, there was a slower accumulation of radiolabel by the parasite, and by 5 minutes the concentration of [¹⁴C]isoleucine inside the cell was approximately 2.3 times higher than that in the extracellular solution.

Depletion of parasite ATP by preincubation of the cells in a glucose-free medium (solution C) had no significant effect on the initial part of the time course [distribution ratio of ~ 0.9 within 15 seconds, initial influx rate of 531 ± 30 nmol/(10¹² cells · h); Figure 2]. However, parasites deprived of glucose failed to
concentrate isoleucine, and in these cells the distribution ratio did not deviate significantly from 0.9 for the duration of the time course.

Suspension of the isolated parasites in a Na⁺-free solution (solution D) had no effect on either the initial influx rate ([507 ± 27 nmol/(10¹² cells • hour)] or the distribution ratio at 5 minutes (2.2 ± 0.1; n = 3). Similarly, decreasing the extracellular pH from 7.3 to 5.5 (in solution E) and thereby imposing a steep inward H⁺ gradient was without significant effect; the influx measured over the first 15 seconds, and the distribution ratio at 5 minutes in cells at pH 5.5 [461 ± 41 nmol/(10¹² cells • hour), 2.4 ± 0.3, respectively] were not significantly different from those in cells at pH 7.3 [600 ± 44 nmol/(10¹² cells • hour), 2.2 ± 0.2, respectively; n = 3; P > .15 in both cases, paired t tests].

The initial component of isoleucine uptake by P. falciparum trophozoites is via a saturable transporter

The data of Figure 2 reveal 2 discrete components to the uptake of isoleucine by isolated parasites. The first, attributed to the initial transport of radiolabel across the parasite plasma membrane, is glucose (and therefore ATP) independent. The second, attributable to the sequestration of radiolabel within the parasite, is glucose dependent.

The kinetics of the initial transport process were investigated by measuring the uptake of isoleucine over 15 seconds into isolated parasites suspended in media having an isoleucine concentration of 0.05 to 10 mM. The concentration dependence of isoleucine influx was resolved into a saturable component (apparent Kₘ of 550 ± 160 μM) and a linear component (Figure 3). Because the 15-second incubation period falls outside the initial linear phase of the uptake time course, the apparent Kₘ estimated here is likely to be an overestimate of the true value.

Isoleucine transport into the parasite is inhibited competitively by leucine

The short-incubation influx experiments giving rise to the data in Figure 3 are technically difficult and are not well suited to testing a large number of experimental conditions. By contrast, measurement of protein synthesis is straightforward. A preliminary assessment of the ability of a range of different amino acids and isoleucine analogs (each at a concentration of 10 mM) to compete with, and thereby inhibit, the uptake of isoleucine was therefore carried out by testing their ability to inhibit the incorporation of [¹⁴C]isoleucine into protein.

The effect of leucine on [¹⁴C]isoleucine influx was tested directly, and compared with the effect of isoleucine. As shown in Figure 4, leucine was an effective inhibitor of isoleucine influx, causing a half-maximal inhibition of the uptake of [¹⁴C]isoleucine when present at 1.19 ± 0.07 mM (n = 4). However, the affinity of the interaction of leucine with the system was somewhat less than that for isoleucine; the concentration of leucine necessary to cause half-maximal inhibition of [¹⁴C]isoleucine uptake was approximately 6 times higher than the concentration of unlabeled isoleucine required to inhibit [¹⁴C]isoleucine uptake to the same extent (0.19 ± 0.03 mM (n = 6); Figure 4).

Isoleucine transport into the parasite is trans-stimulated by intracellular leucine

The experiments reported thus far were carried out under nominally zero-trans conditions, with the parasites largely depleted of intracellular amino acids by preincubation in an amino acid–free medium. In the experiment giving rise to Figure 5A we compared the influx of [¹⁴C]isoleucine measured under nominally zero-trans conditions (in which the isolated parasites were exposed to a 1-mM concentration of isoleucine, leucine, or methionine in the extracellular solution at the time of addition of the radiolabel), with that...
measured under exchange conditions [in which the parasites were preloaded with a 1-mM concentration of the amino acid and therefore had high levels of the amino acid at both the extracellular (cis) and intracellular (trans) faces of the plasma membrane]. The results shown are for ATP-replete parasites; similar results were obtained for parasites that were ATP-depleted (data not shown).

For parasites suspended under nominally zero-trans conditions in medium containing 1 mM methionine [14C]isoleucine equilibrated (ie, the distribution ratio went to ~1) within the 15-second incubation period (Figure 5A, bar E). When either isoleucine or leucine was present (at 1 mM) in the extracellular (but not intracellular) solution, the influx of [14C]isoleucine was slower, reaching distribution ratios of 0.22 ± 0.01 and 0.45 ± 0.06, respectively, within 15 seconds (bars A and C, respectively). This is consistent with isoleucine and leucine competing with [14C]isoleucine for binding to the transporter at the extracellular membrane face. By contrast, when isoleucine or leucine was preloaded into the parasite (in addition to being present in the extracellular solution), [14C]isoleucine influx increased sufficiently for the radiolabel to equilibrate within the 15-second incubation period (bars B and D). Thus, both isoleucine and leucine caused a substantial trans-stimulation of [14C]isoleucine influx.

The trans-stimulation of isoleucine influx by intracellular leucine is consistent with the transporter having the capacity to mediate the exchange of one amino acid for the other. This is demonstrated directly in Figure 5B and C. In an experiment in which isolated, ATP-depleted parasites were preequilibrated with [14C]isoleucine, the addition of 1 mM unlabeled leucine to the extracellular medium resulted in an immediate (transient) efflux of the radiolabel (Figure 5B), consistent with the exchange of extracellular leucine for intracellular [14C]isoleucine. Similarly, in the converse experiment in which parasites were preequilibrated with [14C]leucine, the addition of 1 mM isoleucine to the medium resulted in an immediate (transient) efflux of the radiolabel (Figure 5C), consistent with the exchange of extracellular isoleucine for intracellular [14C]leucine.

The glucose-dependent component of isoleucine uptake is a high-affinity and highly substrate-specific process

The kinetics of the second, glucose-dependent component of isoleucine uptake by isolated parasites were investigated by

---

**Figure 4.** Concentration dependence of the inhibition by leucine and isoleucine of the initial (glucose-independent) uptake of [14C]isoleucine in isolated P falciparum trophozoites, at 20°C. The uptake of [14C]isoleucine was measured over 15 seconds. The extracellular concentration of leucine (C) or isoleucine (○) ranged from 0.1 to 10 mM. Protein synthesis was inhibited by the addition of cycloheximide (40 μM) and anisomycin (150 μM). Both curves were drawn using the following equation: distribution ratio at 15 seconds = AB/(B + [amino acid]) + C fitted to the averaged data by nonlinear least-squares regression. For the inhibition of [14C]isoleucine uptake by leucine: A = 0.89 ± 0.03 (15 seconds)−1 B = 1.2 ± 0.1 mM, and C = 0.012 ± 0.002 (15 seconds)−1 (n = 4), respectively. For the inhibition of [14C]isoleucine uptake by unlabeled isoleucine: A = 0.76 ± 0.03 (15 seconds)−1 B = 0.190 ± 0.001 mM, and C = 0.08 ± 0.01 (15 seconds)−1 (n = 5), respectively. The leucine data are averaged from 4 separate experiments performed on different days and are shown ± SEM. The isoleucine data are derived from those of Figure 3.

**Figure 5.** Trans effects on the transport of [14C]isoleucine and [14C]leucine in isolated P falciparum trophozoites at 20°C. (A) Effect of isoleucine, leucine, or methionine on the initial (glucose-independent) uptake of [14C]isoleucine. Transport was measured over 15 seconds and is expressed in terms of the [14C]isoleucine distribution ratio. Prior to the experiment, isolated parasites were washed twice in amino acid–free saline (solution B), washed once more, then incubated (15 mL; ± 15 minutes; 37°C) in either solution B (for the nominally zero-trans samples) or solution B supplemented with 1 mM unlabeled isoleucine, leucine, or methionine (for the exchange samples). At the end of the incubation period the parasites were washed and resuspended in the same solution supplemented with protein synthesis inhibitors [cycloheximide (40 μM) and anisomycin (150 μM)]. The 15-second uptake measurement commenced with the suspension of the parasites at time zero in a solution containing radiolabeled isoleucine, protein synthesis inhibitors, and a sufficient concentration of the appropriate unlabeled amino acid to give a final extracellular amino acid concentration of 1 mM. The black bars (labeled A, C, and E) indicate the results of the nominally zero-trans experiments in which the unlabeled amino acids were (at time zero) present in the extracellular medium but nominally absent from the parasite cytosol. The gray bars (labeled B, D, and F) indicate the results of the exchange experiments in which the parasites were preloaded with the unlabeled amino acids, and the amino acid was therefore present at similarly high concentrations at both the extracellular (cis) and intracellular (trans) faces of the parasite plasma membrane throughout the 15-second uptake period. The data are averaged from 4 separate experiments performed on different days and are shown ± SEM. (B) Exchange of intracellular [14C]isoleucine for extracellular leucine, isolated parasites depleted of amino acids (as above) were washed twice and incubated (50 mL; 15-20 minutes; 37°C) in glucose-free saline (solution C) to deplete the cells of ATP, then resuspended (for > 10 minutes at 20°C) in solution C supplemented with protein synthesis inhibitors together with [14C]isoleucine to preload the cells with radiolabel. A sample was taken to assess the equilibrium concentration of radiolabel then, at time zero, cell suspension (1.08 mL) was mixed with 10 μL of either solution C (○) or solution C containing 108 mM unlabeled isoleucine to give a final extracellular isoleucine concentration of 1 mM (○). The concentration of radiolabel within the parasite is expressed in terms of the [14C]isoleucine distribution ratio. The data are averaged from 4 separate experiments performed on different days and are shown ± SEM. Asterisks indicate significant difference between the distribution ratios measured in the presence and absence of extracellular amino acid at the different time points, with ** and * denoting P values less than .005 and less than .01, respectively (paired student t test).
measuring the uptake of [14C]isoleucine over an extracellular isoleucine concentration range of 0.5 to 5 μM. Increasing the concentration of unlabeled isoleucine from 0.5 to 5 μM had little effect on the initial influx of [14C]isoleucine (ie, the uptake over 15 seconds; data not shown) but caused a pronounced, concentration-dependent decrease in the accumulation of radiolabel between 15 seconds and 3 minutes (ie, the glucose-dependent component of isoleucine uptake, attributable to the sequestration of radiolabel within the cell), consistent with the process undergoing saturation. Figure 6 shows the concentration-dependence of the rate of this second (glucose-dependent) component of isoleucine uptake. The apparent $K_m$ and $V_{max}$ values, determined for the data from the least-squares fit to the Michaelis-Menten equation, are 0.93 ± 0.26 μM (indicating a very high-affinity process) and 1.73 ± 0.25 μmol/(10¹² cells · hour), respectively.

The substrate specificity of the glucose-dependent component of isoleucine uptake was investigated by testing its susceptibility to inhibition by a range of isoleucine analogs and other amino acids. These included threonine and D-isoleucine, the isoleucine analogs D-alloisoleucine and O-methylthreonine (which both inhibit the incorporation of isoleucine into protein by P. knowlesi), norleucine and norvaline (inhibitors of the Neutral System 1 in plants), and 3-methyl-2-oxopentanoate (the α-keto analog of isoleucine), as well as trifluoroleucine and azaleucine (inhibitors of bacterial isoleucine transporters). The experiments were carried out by measuring the uptake of [14C]isoleucine into isolated parasites at 15 seconds and 3 minutes in the presence of 5 μM of each of the compounds tested. The initial glucose-independent transport of isoleucine (uptake over 15 seconds) was unaffected by the various analogs or amino acids at the low concentration (5 μM) tested here (data not shown). Of all of the compounds tested, only unlabeled isoleucine caused significant inhibition of [14C]isoleucine accumulation over the period 15 seconds to 3 minutes, reducing uptake to 13% ± 7% of the control value ($P = .005$).

**Discussion**

**Transport of isoleucine into P. falciparum–infected erythrocytes**

In this study we have investigated isoleucine transport in both normal and P. falciparum–infected human erythrocytes. Consistent with a previous report, isoleucine uptake by normal erythrocytes was found to be rapid and nonconcentrative (reaching a final distribution ratio of ~ 0.9; Figure 1A). Infection by P. falciparum caused a 5-fold increase in the rate of isoleucine influx into human erythrocytes. This increase in transport was wholly blocked by furosemide, consistent with it being via the parasite-induced NPPs which, under physiologic conditions, serve as the major route for the uptake of isoleucine into infected cells, accounting for approximately 80% of influx (as represented schematically in Figure 7). Past years have seen a spate of studies on the transport and electrophysiologic characteristics of these pathways; however, their origin and identity remain unknown.

Infected erythrocytes accumulated [14C]isoleucine to significantly higher levels than uninfected erythrocytes (Figure 1A), consistent with the observation that isolated parasites accumulated [14C]isoleucine (via an ATP-dependent mechanism; Figure 2).

Our finding of a marked increase in the permeability of erythrocytes to isoleucine on P. falciparum infection is consistent with the findings of Ginsburg et al but contrasts with the report from Elford et al that there was no such change.

**The role of the NPPs in the synthesis of protein by the intracellular parasite**

P. falciparum–infected erythrocytes incorporated isoleucine into protein at a rate of approximately 170 μmol/(10¹² cells · hour), and, although this rate is well below (and should therefore be supported comfortably by) the overall rate of isoleucine transport into parasitized cells (~ 550 μmol/(10¹² cells · hour)), it is significantly higher than the rate of isoleucine influx via endogenous mechanisms alone (~ 110 μmol/(10¹² cells · hour)). The data are therefore consistent with the influx of isoleucine via the NPPs serving to prevent parasite protein synthesis from being rate-limited by the transport of this essential amino acid across the host.
cell membrane. Consistent with this, inhibition of the NPPs with furosemide significantly reduced the incorporation of exogenous isoleucine by infected erythrocytes (Figure 1C). By contrast, furosemide had no effect on isoleucine incorporation by isolated parasites, indicating that the effect of furosemide on protein synthesis in parasitized erythrocytes is attributable to the transport of the compound at the erythrocyte membrane and not to an effect on the translation machinery within the parasite.

It should be noted that when the NPPs were blocked by furosemide, protein synthesis in the infected cell occurred at only approximately 35% of the normal rate, despite the fact that under these conditions the rate of isoleucine influx via the endogenous pathways is approximately 65% of the normal rate of protein synthesis, and protein synthesis might therefore be expected to proceed at 65% of its normal rate. These data might be explained if, in addition to isoleucine, the NPPs mediate the influx (nutrient) or efflux (waste product) of other solute(s) that influence parasite protein synthesis. Because the inhibitory effect of furosemide was apparent within 15 seconds, and the time course was linear thereafter (Figure 1C), it is unlikely that the reduction in protein synthesis was a consequence of parasite deterioration caused by the build-up of a toxic waste product (e.g., lactic acid) in the infected erythrocyte. Rather, it is more likely that furosemide blocked the uptake of an exogenous nutrient required by the parasite for protein synthesis (e.g., another amino acid).

Transport of isoleucine into the parasite

The uptake of isoleucine by the intracellular parasite itself was resolved into 2 discrete components: an initial, rapid ATP-independent component, and a more gradual process which sequenced into 2 discrete components: an initial, rapid ATP-dependent component of isoleucine influx via the endogenous pathways (at concentrations of isoleucine within the parasite via an ATP-dependent mechanism (Figure 2). The initial ATP-independent (ion-independent) component of isoleucine uptake can be attributed to the transport of the amino acid into the parasite, across the parasite plasma membrane, and was composed of both saturable \( K_{m} = 550 \mu M \) and linear \( k_{d} = 0.3 L/(10^{12} \text{ cells} \cdot \text{hour}) \) transport components (Figure 3). Although the linear component contributed significantly to transport at high (supraphysiologic) concentrations of isoleucine, the saturable component of transport was the major mode of isoleucine entry into the parasite at isoleucine concentrations within the normal physiologic range (see Figure 3 inset). The linear component of isoleucine influx may represent passive diffusion of the amino acid across the plasma membrane, although low-affinity transport via other systems cannot be ruled out.

The finding that leucine is a substrate for the isoleucine transporter, and, in particular, that the transporter has the capacity to exchange intracellular leucine for extracellular isoleucine (Figure 5C), is of particular interest from a physiologic point of view. Leucine is (together with alanine) the most abundant amino acid residue in adult human hemoglobin and is therefore a major product of the parasite’s digestion of host hemoglobin. Thus, under physiologic conditions the parasite transporter characterized here may effectively serve a number of roles, catalyzing the efflux from the parasite cytosol of leucine (and perhaps valine and phenylalanine, the other 2 amino acids implicated as interacting with the transporter and both of which are abundant in adult hemoglobin) in exchange for isoleucine, the one amino acid that the parasite is unable to obtain from hemoglobin digestion (Figure 7).

ATP-dependent accumulation of isoleucine within the parasite

The ATP-dependent component of isoleucine uptake by the parasite was via a high affinity \( K_{m} = 0.9 \mu M \), low velocity \( V_{max} = 1.7 \mu mol/(10^{12} \text{ cells} \cdot \text{hour}) \) and highly substrate-specific mechanism and generated an accumulation of radiolabel inside the cell, perhaps through the active (energy-dependent) uptake of isoleucine into an intracellular compartment. Alternatively, or in addition, the ATP-dependent accumulation of isoleucine may be the result of the metabolic trapping of isoleucine derivatives within the parasite. The degradation of isoleucine begins with its transamination to 3-methyl-2-oxopentanoate, which is then oxidatively decarboxylated to produce 2-methylbutanoyl-CoA, and then continues along the fatty acid oxidation pathway to generate acetyl CoA and the succinyl-CoA precursor propionyl CoA. To date, putative homologs of the first 2 enzymes of this pathway have been identified in the \( P. falciparum \) genome. These are a branched chain amino acid dehydrogenase (PF140286\(^{38,39}\)), predicted to be localized to the parasite’s apicoplast,\(^{40}\) and the α and β subunits of a 3-methyl-2-oxobutanoate dehydrogenase (PF13_0070 and PFE0225w, respectively\(^{39}\)), predicted to be targeted to the mitochondrion.\(^{41}\)

We have recently described the presence of genes for 6 putative amino acid transporters in the \( P. falciparum \) genome (one of which, PFL1515c, is predicted to be targeted to the apicoplast), as well as a possible candidate for the NPPs.\(^{42}\) All are expressed (at the mRNA level) during the intraerythrocytic cycle.\(^{42}\) Which of these might play a role in the processes characterized here is the subject of ongoing study.

Acknowledgments

We thank the Canberra Branch of the Australian Red Cross Blood Service for the provision of blood and Rosa Marchetti for culturing the parasites used in the experiments giving rise to Figure 5B and C. This work was supported by the Australian Research Council (grant DP0559433).

Authorship

Contribution: R.E.M. and K.K. designed the research and wrote the paper. R.E.M. performed the research and analyzed and interpreted the data.

Conflict-of-interest disclosure: The authors declare no competing financial interests.

Correspondence: Kiaran Kirk, School of Biochemistry and Molecular Biology, The Australian National University, Canberra, A.C.T. 0200, Australia; E-mail: kiaran.kirk@anu.edu.au

References


Transport of the essential nutrient isoleucine in human erythrocytes infected with the malaria parasite *Plasmodium falciparum*

Rowena E. Martin and Kiaran Kirk