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Characterisation of exogenous folate transport in *Plasmodium falciparum*

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Abstract

Folate salvage by *Plasmodium falciparum* is an important source of key cofactors, but little is known about the underlying mechanism. Using synchronised parasite cultures, we observed that uptake of this dianionic species against the negative-inward electrochemical gradient is highly dependent upon cell-cycle stage, temperature and pH, but not on mono- or divalent metal ions. Energy dependence was tested with different sugars; glucose was necessary for folate import, although fructose was also able to function in this role, unlike sugars that cannot be processed through the glycolytic pathway. Import into both infected erythrocytes and free parasites was strongly inhibited by the anion-channel blockers probenecid and furosemide, which are likely to be acting predominantly on specific folate transporters in both cases. Import was not affected by high concentrations of the antifolate drugs pyrimethamine and sulfadoxine, but was inhibited by the close folate analogue methotrexate. The pH optimum for folate uptake into infected erythrocytes was 6.5–7.0. Dinitrophenol and nigericin, which strongly facilitate the equilibration of H^+ ions across biological membranes and thus abolish or substantially reduce the proton gradient, inhibited folate uptake profoundly. The ATPase inhibitor concanamycin A also greatly reduced folate uptake, further demonstrating a link to ATP-powered proton transport. These data strongly suggest that the principal folate uptake pathway in *P. falciparum* is specific, highly regulated, dependent upon the proton gradient across the parasite plasma membrane, and is likely to be mediated by one or more proton symporters.
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Keywords: Folate metabolism; Folate salvage; Malaria parasites; Metabolic inhibitors; Proton symport; Transporters

1. Introduction

Reduced folates are essential cofactors for one-carbon transfer reactions, including the conversion of dUMP to dTMP, which is a prerequisite for DNA synthesis. Because of this, the folate pathway has long been a target for drugs deployed against rapidly reproducing cells such as cancers and a range of microbial pathogens. Whereas most microorganisms can synthesise the folates they need from the simple precursors GTP, *p*-aminobenzoic acid (pABA) and glutamate, higher animals (but not plants) have lost the ability to do this and depend on dietary intake of pre-formed folate as an essential nutrient. The human malaria parasite *Plasmodium falciparum* is able to exploit both of these routes [1–4]. Thus, it can utilise folate provided in culture medium in vitro or salvaged from the host plasma in vivo on the one hand, or convert the above precursors de novo into folate derivatives on the other, a characteristic also shared by the related apicomplexan parasite *Toxoplasma gondii* [5,6]. The relative importance of the biosynthetic and salvage pathways across the complete life cycle in vivo and the interplay between them is poorly understood, although existing data support the view that both are necessary for healthy propagation of the parasite, at least in the erythrocytic stages [7].

An important aspect of exogenous folate utilisation is the machinery and mechanism(s) by which folate is imported into the parasite. The highly polar nature of folate derivatives sug-

Abbreviations: BCECF-AM, 2′,7′-bis-(2-carboxyethyl)-5,6-carboxylfluorescein acetoxymethyl ester; DHFR, dihydrofolate reductase; DHPS, dihydropteroate synthase; DNP, 2,4-dinitrophenol; pABA, *p*-aminobenzoic acid; MTX, methotrexate; NPP, new permeability pathway(s); PBS, phosphate-buffered saline; PPPK, 6-hydroxymethylpterin pyrophosphokinase; PYR, pyrimethamine; SDX, sulfadoxine; THF, tetrahydrofolate; TS, thymidylate synthase

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gests that salvage must employ some kind of mediated transport process, as diffusion alone across the membrane is likely to be far too inefficient. Moreover, folate molecules are dianionic at physiological pH and must be imported into the parasite against an inwardly negative electropotential that has been measured as ca. −95 mV in *P. falciparum* [8]. Although the transport of other key molecules, such as pantothenate, lactate, glucose and choline, has been investigated [9–13], there has been no detailed study to date of this aspect of folate metabolism in *P. falciparum*, a better understanding of which might lead to new ways of inhibiting parasite growth. A preliminary approach to this end has been taken by demonstrating that the anti-gout drug, probenecid, which among other things inhibits folate transport in mammalian cells [14], can increase the sensitivity of *P. falciparum* to antifolate inhibitors [15]. Here, we demonstrate that folate uptake by this parasite is a regulated process that is critically dependent upon provision of glucose or another sugar that can proceed through the glycolytic pathway, that the existence of a pH gradient across the plasma membrane is also required for efficient transport, and that folate is transported principally by a proton-symport mechanism.

2. Materials and methods

2.1. Chemicals

[3′,5′,7,9-^{3}H]folic acid, 24 Ci mmol^{-1}, 1 mCi ml^{-1} was from Amersham, UK, [3′,5′,7,9-^{3}H]folinic acid (26 Ci mmol^{-1}, 1 mCi ml^{-1}) and [3′,5′,7,9-^{3}H]5-methyltetrahydrofolic acid (44 Ci mmol^{-1}, 1 mCi ml^{-1}) were both from Moravek, California. Folic acid, folinic acid, 5-methyltetrahydrofolic acid, 2,4-dinitrophenol, concanamycin A, D- and L-glucose, D-fructose, D-xylose, D-galactose, 6-deoxy-D-glucose, probenecid and furosemide were all purchased from Sigma, UK. Note that we use the term 'folate' generically to indicate derivatives of the folate family of molecules regardless of their oxidation state, modifications at the 5 and 10 positions or polyglutamation status.

2.2. Parasite culture

P. falciparum was routinely cultured under 1% O_2, 3% CO_2, 96% N_2 in RPMI 1640 medium, supplemented with D-glucose (22 mM final concentration), hypoxanthine (36 mM), HEPES (25 mM), gentamicin sulfate (50 μg/ml) and 0.5% Albumax II (Invitrogen). The cultures were synchronised by haemolysis of mature, late trophozoite-stage parasitised erythrocytes by suspension in 9 volumes of a 5% sorbitol solution at room temperature for 5 min. Cells surviving the treatment were used to set up new cultures and the process repeated where necessary to achieve a tighter synchronisation [16]. Given that synchrony is never perfect, time zero for the erythrocytic cycle was taken as the point where ca. 90% of infected erythrocytes were ring form, with the remainder as very late schizonts. To obtain free parasites, synchronised cultures, normally in the late (mature) trophozoite stages (ca. 30 h into the cycle), were quickly lysed with 0.05% (w/v) saponin at room temperature, permeabilising the red cell and parasitophorous membranes and leading to haemolysis [17,18]. Any remaining unlysed red cells were further treated with a PBS wash containing the same amount of saponin. The freed parasites were then resuspended in the appropriate buffer with or without glucose, depending upon the purpose of the assays. Saponin-freed parasites that are glucose-replete maintain intracellular levels of ATP for periods of at least 30 min and do not show evidence of leakage [19].

2.3. Uptake assay of radiolabelled folates

Folate uptake/export assays were performed on free parasites, parasitised red cells or uninfected red cells, as appropriate. Cell numbers were estimated with a haemocytometer. Normally preparations containing 10^7–10^8 parasites were used for each assay point (or ca. 10^7–10^8 uninfected red cells). Prior to uptake assays by naked parasites, infected cells were lysed in 0.05% saponin and the freed parasites washed either in PBS or folate/pABA depleted RPMI 1640, depending upon the experiment. Wash steps were also performed with different buffered or non-buffered isotonic salt solutions where necessary as indicated in the relevant text.

All cells, washed extensively to remove folate present in the culture medium, were mixed with or without glucose (20 mM final concentration) and inhibitors as appropriate, together with radiolabel (normally 1 μCi/ml, equivalent to 38 nM, unless otherwise stated) to make up a total volume of 100 μl with PBS or alternative buffer. All the components were prewarmed to 37 °C before the start of the assay, the reaction mix incubated at 37 °C normally for 30 min, and stopped by addition of 1 ml ice-cold PBS.

When assaying for folate uptake by whole parasitised red cells, these were spun down after the uptake period and the pellet washed with 1 ml ice-cold PBS at least three times to bring extracellular label down to the background level. The washed parasite pellet obtained by subsequent saponin treatment was then lysed with 0.02% SDS before counting. Uptake by naked parasites was determined in the same way. Unless otherwise indicated, the uptake values represent labelled folate that has been imported into synchronised trophozoite stage parasites. All assays were routinely performed in triplicate on different batches of parasites unless otherwise indicated, and the data expressed as the mean ± S.D.

2.4. Extraction and affinity purification of folate derivatives

For characterisation of labelled folates, parasite pellets were washed three times in 1 ml PBS to remove any extracellular label and resuspended in 1 ml extraction buffer (0.1 M Tris–HCl, 2% ascorbic acid, pH 7.5), heated in a boiling water bath for 10 min, centrifuged at 10,000 × *g* for 10 min and supernatants stored at −20 °C until affinity purification and HPLC analysis, performed as described [20]. Unlabelled folic acid, folinic acid and PteG2 to PteG5 of the polyglutamated forms of folic acid were added into the extracted folates as internal standards. An identical aliquot of sample was also spiked with ^{3}H-folinic acid in a second run to confirm the identity of the

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