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Review

Central carbon metabolism of *Plasmodium* parasites

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ABSTRACT

The central role of metabolic perturbation to the pathology of malaria, the promise of antimetabolites as antimalarial drugs and a basic scientific interest in understanding this fascinating example of highly divergent microbial metabolism has spurred a major and concerted research effort towards elucidating the metabolic network of the *Plasmodium* parasites. Central carbon metabolism, broadly comprising the flow of carbon from nutrients into biomass, has been a particular focus due to clear and early indications that it plays an essential role in this network. Decades of painstaking efforts have significantly clarified our understanding of these pathways of carbon flux, and this foundational knowledge, coupled with the advent of advanced analytical technologies, have set the stage for the development of a holistic, network-level model of plasmodial carbon metabolism. In this review we summarize the current state of knowledge regarding central carbon metabolism and suggest future avenues of research. We focus primarily on the blood stages of *Plasmodium falciparum*, the most lethal of the human malaria parasites, but also integrate results from simian, avian and rodent models of malaria that were a major focus of early investigations into plasmodial metabolism.

Contents

1. Introduction 95
2. Glycolysis 96
3. Other carbon sources 96
4. Pentose phosphate pathway 98
5. Glycosylation and aminosugars 98
6. Lipid biogenesis 98
7. Tricarboxylic acid metabolism 99
8. Systems level studies 100
9. Suggested research avenues and concluding remarks 101
References 101

1. Introduction

While the process of evolving into a parasitic niche seems to have resulted in a 'paring down' of many *Plasmodium* metabolic pathways, including the wholesale loss of *de novo* amino acid and purine biosynthesis, pioneering early work suggested that most of the core conserved components of carbon metabolism – glycolysis [1,2], the pentose phosphate pathway [3], lipid biogenesis [4,5], glycosylation [6,7] and at least some components of citric acid metabolism [8,9] – were present in some form. However, efforts to precisely map the flow of carbon through the metabolic network of the malaria parasite were often stymied by difficulties pertaining to the isolation and culturing of parasites, complications arising from the interconnected nature of parasite and host cell metabolism, technical limitations of metabolic tracing using classical methodologies, and in some cases, marked divergence between pathways in *Plasmodium* spp. and the model organisms in which they were first elucidated (reviewed in [10–12]). Dedicated efforts by a host of researchers, however, have resolved most of these technical problems and filled in many of the blank spots in the metabolic map.

Abbreviations: EMP, Embden–Meyerhof–Parnas; RBC, red blood cell; PEP, phosphoenolpyruvate; PEPCK, PEP carboxykinase; PEPC, PEP carboxylase; PPP, pentose phosphate pathway; G6PDH, glucose-6-phosphate dehydrogenase; GPI, glycophosphatidylinositol; ENR, enoyl-acyl carrier protein reductase; PDH, pyruvate dehydrogenase; TCA, tricarboxylic acid; IDH, isocitrate dehydrogenase; NMR, nuclear magnetic resonance; GSH, reduced glutathione; GSSG, oxidized glutathione (glutathione disulfide).

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The picture that arises from these studies is of a network that is both more streamlined and more modular than that found in free-living protozoa. This seems to be a consequence of dispensing with the flexibility that a free-living microbe must maintain in order to dynamically regulate its metabolic network to consume a wide variety of possible combinations of nutrients. Within the nutrient-rich and homeostatic environment of the blood stages, the parasite appears to consume a defined set of nutrients (glucose, glutamine, free fatty acids, etc.) *via* several discrete pathways (glycolysis/pentose phosphate pathway, carboxylic acid metabolism, fatty acid elongation and modification) with a low degree of interconnectivity. A fine-grained understanding of plasmodial metabolism will hopefully aid in exploiting this metabolic rigidity when selecting drug targets and designing antimalarials.

We expect the broad outline of central carbon metabolism to be conserved between the *Plasmodium* spp. due to the markedly similar life cycles, comparable drug sensitivities and low levels of divergence in metabolic enzymes at the genome level. However, we caution that the metabolism of the rodent and avian malaria parasites in particular may well be more complicated given their proclivity to invade immature, nucleated erythrocytes (reticulocytes) that are more metabolically complex than the mature erythrocytes favored by simian parasites.

2. Glycolysis

Based on numerous classical experiments, carbon metabolism of the malaria parasites has been considered largely synonymous with carbohydrate metabolism, principally the Embden–Meyerhof–Parnas (EMP) pathway of glycolysis. It has long been clear from *in vitro* studies of blood-stage *Plasmodium* parasites that they are voracious consumers of glucose (blood sugar). The host cell, at least in the human parasites, is the mature erythrocyte (red blood cell, or RBC), whose metabolism has been exhaustively studied and is comparatively simple enough to be simulated by comprehensive kinetic models [13,14]. The RBC lacks a mitochondrion and therefore is entirely dependent on glucose fermentation. As a nonproliferative cell with modest energetic needs, RBC glucose consumption is relatively low, on the order of 5 μmol glucose/24 h/10^9 RBCs [15]. Upon invasion by a *Plasmodium* parasite, however, the glucose consumption rate is estimated to increase up to 100-fold at the most metabolically active trophozoite and schizont stages [12].

In vitro culture experiments using several other sugars have established that, besides glucose, only fructose can support continuous growth (albeit at a reduced rate) [16,17]. Most of the glucose consumed (60–70%) by *Plasmodium falciparum* is incompletely oxidized to lactic acid and excreted [15], although the exact percentage varies between different *Plasmodium* species and the atmospheric culture conditions used. This glucose consumption contrasts with the >90% glucose-to-lactate conversion observed in uninfected RBCs and reflects the increased flux of glucose carbon into biomass (nucleic acids, lipids, glycosylated proteins) required for proliferating parasites. Only a very small fraction of the total glucose is completely oxidized to carbon dioxide, at least in mammalian malaria parasites [1,18,19], which has generally been taken to indicate the absence of a functional citric acid cycle contributing to energy generation. This is in keeping with the observation that *in vitro* cultures of *P. falciparum* exhibit only minimal oxygen consumption [20] (and in fact prefer microaerophilic conditions of ~5% oxygen, being growth-inhibited by normal atmospheric oxygen concentrations [21].) These results strongly suggest that blood-stage *Plasmodium* rely primarily upon glucose fermentation for their energetic needs. Accordingly, inhibitors of mitochondrial respiration have only a small effect on parasite ATP pools [22].

The parasite accommodates this vastly increased need for glucose by expressing at least one essential hexose transporter to the surface of the infected cell [23], increasing the hexose permeability of the erythrocyte membrane [24]. Such modifications of the host cell raise the question of precisely how the host glycolytic machinery interacts with that of the parasite. All the enzymes required for a complete EMP pathway are (1) encoded in the parasite genome [25], (2) expressed during the blood stages [26], and (3) detected in infected cells, in some cases substantially increasing the activity normally observed in RBCs (reviewed in [27]). Free glucose should be quickly phosphorylated to glucose-6-phosphate by the host cell hexokinase upon entry into the RBC cytosol, rendering it membrane-impermeant and effectively trapping it within the host compartment. Its import into the parasite may depend on dephosphorylation by an acid phosphatase (PFI0880c) that is trafficked to the surface of the parasite plasma membrane and cleaves phosphate from a diversity of small molecules [28]. This nonspecific cleavage of high-energy phosphate bonds might have the effect of draining energy from the host cytosol, despite the fact that the parasite seems to require the host cell to remain "viable" (maintaining its redox state, etc.) until lysis occurs in order to successfully complete its developmental cycle. Malaria parasites may circumvent this difficulty by actively supplying the host with metabolically useful molecules such as ATP [29] and glutathione [30]. This secreted phosphatase may also help explain the decline observed in infected cells in the levels of 2,3-diphosphoglycerate [31–33], an allosteric regulator of hemoglobin oxygen affinity produced by an enzyme (diphosphoglycerate mutase) present in the erythrocyte but not the parasite [25,34].

3. Other carbon sources

Many microbes that prefer sugars as a carbon source maintain the ability to metabolize other compounds such as acetate, pyruvate, ethanol or amino acids depending on their availability. Generating the 5- and 6-carbon sugars necessary for growth then depends on gluconeogenesis, in which the catabolic reactions of glycolysis are essentially reversed through the use of different enzymes at the regulated thermodynamic control steps (reviewed in [35]). However, the complete sequencing of the *P. falciparum* genome revealed no homologs of fructose bisphosphatase, an enzyme normally required for gluconeogenesis [25]. Strangely, the parasite does possess phosphoenolpyruvate carboxykinase (PEPCK, PF13_0234) [36], an enzyme converting oxaloacetate and ATP to PEP and carbon dioxide that usually functions in supplying citric acid cycle or amino acid-derived carbon to gluconeogenesis. The parasites also encode a PEP carboxylase (PEPC, PF14_0246), which has almost the opposite gene expression profile (peak expression at 18 and 47 h post-invasion for PEPCK and PEPC, respectively) [26]. PEPC has been purified from *Plasmodium berghei* [37], and essentially runs the reverse reaction: PEP and carbon dioxide converted to oxaloacetate and inorganic phosphate. The role of this enzyme remains unclear, but would suggest a regulated carbon fixation step.

It is well-established through $^{14}CO_2$ incorporation experiments that all studied *Plasmodium* spp. possess the ability to fix carbon, although the nature of the end-products differs between species [11]. Our own metabolomic investigation of carbon flux in *P. falciparum* showed clear evidence of PEPC activity, as parasites fed uniformly ^{13}C-labeled glucose generated oxaloacetate/malate labeled at three of the four carbons (indicative of the carboxylation of PEP) [38] (Fig. 1). The purpose of this side pathway is unclear

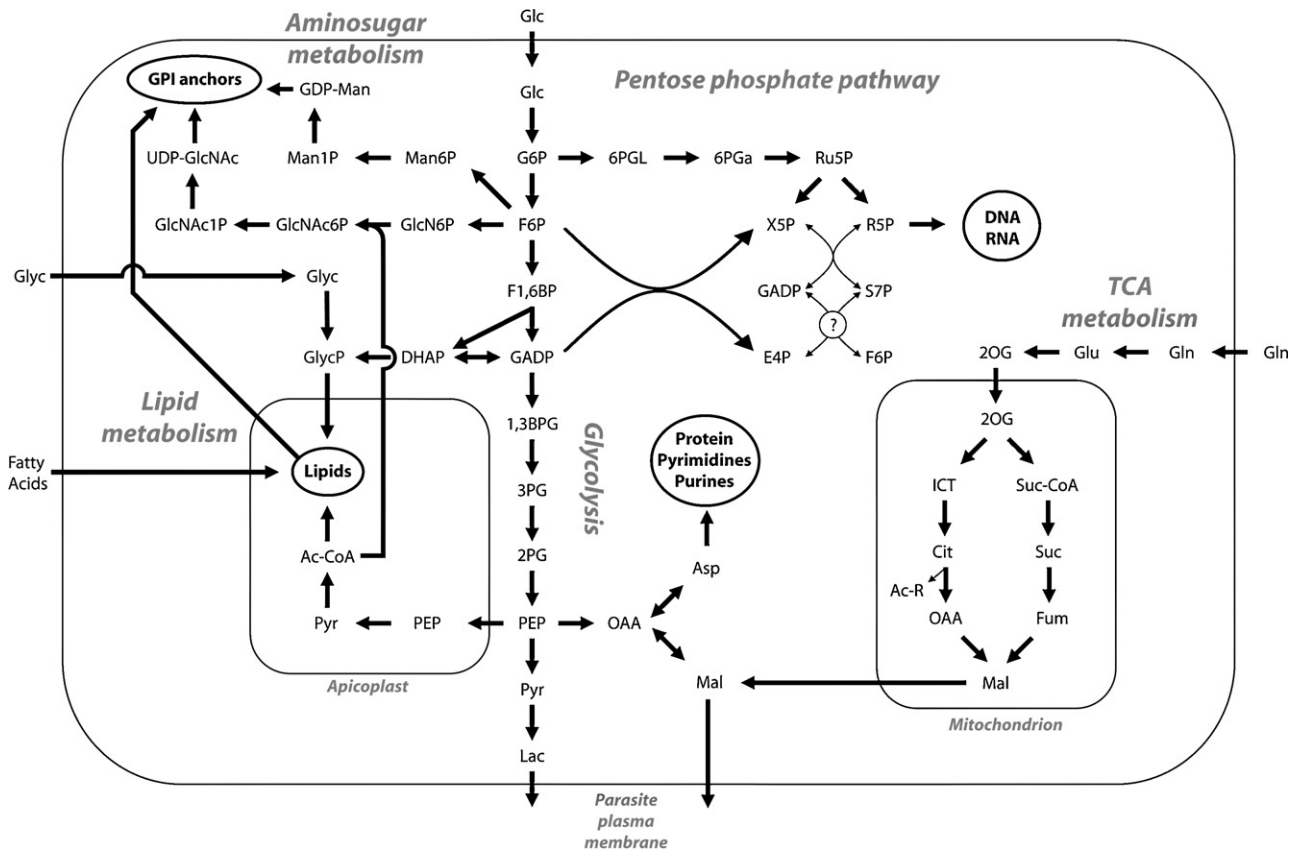

Fig. 1. An integrated map of carbon flow through the metabolic network of *Plasmodium falciparum*. Arrows show the proposed direction of flux through the corresponding enzymatic reaction as suggested by experimental evidence; note that this is only intended to indicate net flux, and that the reaction in question might be reversible. Cofactors (ATP, NADH, etc.) are not shown for the sake of clarity. Text in circles represent major biomass components; the circled question mark indicates uncertainty about the existence of the enzyme transaldolase. *Abbreviations*: Glc, glucose; G6P, glucose-6-phosphate; F6P, fructose-6-phosphate; F1,6BP, fructose-1,6-bisphosphate; DHAP, dihydroxy-acetone-phosphate; GADP, glyceraldehyde-3-phosphate; 1,3BPG, 1,3-bisphosphoglycerate; 3PG, 3-phosphoglycerate; 2PG, 2-phosphoglycerate; PEP, phosphoenolpyruvate; Pyr, pyruvate; Lac, lactate; Ac-CoA, acetyl-CoA; Ac-R, either acetate or acetyl-CoA; GlycP, glycerol-3-phosphate; Glyc, glycerol; Man6P, mannose-6-phosphate; Man1P, mannose-1-phosphate; GDP-Man, GDP-mannose; GlcN6P, glucosamine-6-phosphate; GlcNAc6P, N-acetyl-glucosamine-6-phosphate; GlcNAc1P, N-acetyl-glucosamine-1-phosphate; UDP-GlcNAc, UDP-N-acetyl-glucosamine; 6PGL, 6-phosphoglucono-δ-lactone; 6PGa, 6-phosphogluconate; Ru5P, ribulose-5-phosphate; R5P, ribose-5-phosphate; X5P, xylulose-5-phosphate; S7P, sedoheptulose-7-phosphate; E4P, erythrose-4-phosphate; Asp, aspartate; Gln, glutamine; Glu, glutamate; 2OG, 2-oxoglutarate; ICT, isocitrate; Cit, citrate; OAA, oxaloacetate; Mal, malate; Suc-CoA, succinyl-CoA; Suc, succinate; Fum, fumarate; GPI, glycophosphatidylinositol.

since we have found that the majority of the malate produced in this fashion is excreted from the cell. This results in an energetic cost of one ATP molecule for every molecule of PEP that does not complete glycolysis. Perhaps PEPC acts *in vivo* to supply oxaloacetate as a carbon skeleton for the generation of aspartate by aspartate transaminase (PFB0200c) when aspartate concentrations are limiting. However, in cell culture, aspartate levels are relatively high and this flux may be redirected to malate, which is then excreted due to overflow metabolism. In our studies, aspartate was clearly interconverting with cytosolic oxaloacetate/malate pools, although we were unable to detect any gluconeogenic PEPCK activity in the asexual stages, since ^{13}C-labeled aspartate failed to label PEP, pyruvate or lactate [38]. Interestingly, the up-regulation of PEPCK in *P. falciparum* gametocytes and zygotes has prompted the hypothesis that there is a switch to gluconeogenic metabolism at these stages [36]. How this can be achieved in the absence of fructose bisphosphatase remains unclear.

Several early reports suggested that various *Plasmodium* spp. possess at least a limited ability to metabolize a number of other substrates, such as glycolytic end-products or tricarboxylic acid cycle intermediates [39–42]. However, most of these experiments rely on the stimulation of oxygen uptake as a measure of nutrient consumption, which can be problematic given the unclear relationship between this metric and parasite growth. Possible contaminating sources for this activity have been extensively discussed elsewhere [10–12]. Other experiments using simian and avian malaria parasites directly demonstrated the conversion of radioactively labeled lactate and pyruvate to glycolytic intermediates, organic acids and volatiles such as formate and acetate [1,9,39]. However, we caution that since these experiments used non-physiological glucose-free conditions and/or involved erythrocyte-free parasite preparations, special care should be taken in weighing their relationship to *in vivo* parasite metabolism, where (1) the parasite resides within a selectively permeable host erythrocyte, (2) serum glucose levels are robustly maintained by the host, and (3) lactate is rapidly excreted.

Plasmodium spp. also lack the ability to generate carbohydrate stores in the form of polysaccharides such as glycogen [18,19,43]. Thus the blood-stage parasites are obligately dependent on the fermentation of a constant and abundant supply of glucose. This adaptation is sensible given the parasite's adaptation to a peculiar evolutionary niche: glucose is the most abundant nutrient in human serum and its homeostasis is maintained by a powerful regulatory system. The availability of carbohydrates or other potential carbon sources in the other stages of the life cycle is difficult to study given the general intractability of culturing these stages, but it has been shown that the hemolymph of *Anopheles stephensi* is rich in glucose and the storage carbohydrate trehalose [44] and the essential *P. berghei* hexose transporter (PB000562.01.0) is expressed throughout development in the mosquito [23].

4. Pentose phosphate pathway

The pentose phosphate pathway (PPP, also known as the hexose monophosphate shunt), a critical conserved pathway in virtually all cells capable of metabolizing carbohydrates as a carbon source, is composed of two interconnected branches. The oxidative arm, in which glucose-6-phosphate is ultimately oxidized to ribose-5-phosphate, generates both the riboses needed for nucleic acid synthesis as well as NADPH, which is used for redox control and as a cofactor for biosynthetic reactions. The non-oxidative arm, comprising a series of reversible reactions interconverting 3, 4, 5, 6 and 7-carbon sugar phosphates can either recycle ribose-5-phosphate generated by the oxidative arm back into glycolytic intermediates (when NADPH is required but nucleotide synthesis is not) or else converts glycolytic intermediates into ribose-5-phosphate without concomitant NADPH production (Fig. 1). It is one of the major metabolic pathways in human erythrocytes, consuming 3–11% of the glucose metabolized under normal conditions [45], as it is the only source of the NADPH required to reduce glutathione in response to oxidative stress. Though counterintuitive given its indispensable nature in proliferating cells, the existence of a complete PPP in the malaria parasites was for decades a point of controversy due to difficulties in detecting the necessary enzymes in parasite extracts [11] and early reports of very slight increases in pathway activity in RBCs infected with simian, avian and rodent malaria parasites [3,9,19]. However, the presence of the first and rate-limiting enzyme in the pathway, glucose-6-phosphate dehydrogenase (G6PDH, PF14_0511), was eventually established [46,47]. The remaining enzymes are also encoded in the genome [25] and expressed [26], with the singular exception of transaldolase, for which no homolog has yet been identified [25].

The infected RBC commits roughly the same fraction of glucose to the oxidative PPP as does a normal RBC [48]. Since glucose consumption is massively increased during infection [12], the absolute flux through the PPP is likewise increased. The most recent investigation of this pathway in *P. falciparum* determined that the activity of the oxidative branch increased 78-fold by the trophozoite stages [48]. 82% of this activity could be recapitulated in free parasites, indicating that the parasite is responsible for the majority of the flux but also that the erythrocyte PPP is up-regulated 24-fold, to levels roughly similar to those observed when subjecting uninfected RBCs to oxidative insult. Two reports, one using *P. falciparum* [48] and another using the simian parasite *Plasmodium knowlesi* [3], have found that significant amounts of radioactivity are incorporated into parasite nucleic acids from both 1- and 6-^{14}C-glucose. Since the carbon at position 6 is lost to decarboxylation in the oxidative PPP and so cannot be incorporated into ribose, this implies that the non-oxidative PPP (in which glycolytic intermediates can be converted to riboses) contributes significantly to the total pool of ribose-5-phosphate.

Despite the observation that the parasite PPP predominates in terms of flux, clear evidence for the importance of the host pathway is found in the fact that G6PDH deficiency, the most common human enzymopathy, is associated with resistance to clinical malaria (reviewed in [49]). The mechanistic basis for this protection remains somewhat controversial; in some cases slowed growth in G6PDH-deficient erythrocytes has been directly observed *in vitro* [50], while another group disputes this claim and suggests that the protection is mediated instead by an increase in oxidative damage to the host cell membrane, marking it for early phagocytosis by macrophages [51]. Nevertheless, the parasite requires a certain degree of viability from its host cell during the progression through blood-stage development, which includes maintenance of an adequate glutathione pool, with a sufficiently high ratio of reduced (GSH) to oxidized glutathione (GSSG, glutathione disulfide), within the host cell cytosol [52]. The regeneration of GSH from GSSG is fueled by NADPH from the RBC's pentose phosphate metabolism, whose flux is limited by G6PDH. Intriguingly, it has been reported that the parasite also actively supplies GSH to the host compartment while inducing the excretion of GSSG [30], suggesting that a combined effort encompassing both recycling by the host and biosynthesis by the parasite may be required to sustain the infected cell.

5. Glycosylation and aminosugars

The malaria parasite expresses a variety of glycoconjugated proteins (circumsporozoite protein, the merozoite surface proteins), several of which are essential for invasion and virulence [53]. However, the range of glycoconjugates produced, and the corresponding biosynthetic enzymes, appears to be severely restricted, limited almost entirely to the production of glycophosphatidylinositol (GPI) anchors required for protein-membrane association (reviewed in [54]). These moieties are assembled stepwise in the lumen of the endoplasmic reticulum on a phosphatidylinositol core through the sequential addition of glucosamine (as its activated form, UDP-N-acetyl-glucosamine, subsequently deacetylated) and four mannose molecules. The inositol is acylated, typically by myristic acid, at the 2-O position prior to mannosylation; the third mannose in the chain serves as the site for addition of ethanolamine phosphate, through which the GPI anchor is then attached to a protein.

This pathway thus draws carbon compounds from a variety of sources: hexose sugars (mannose, the glucose moiety of glucosamine), inositol biosynthesis, lower glycolysis (glycerol phosphate from the reduction of dihydroxy-acetone-phosphate, acetyl-CoA from the oxidation of pyruvate), ethanolamine from the host, and lipids from scavenging and modification (Fig. 1). We have recently shown that the acetyl moieties utilized for the synthesis of UDP-N-acetyl-glucosamine ultimately derive from glucose, presumably through the action of the pyruvate dehydrogenase (PDH) complex on glycolytic pyruvate [38]. Thus it is surprising that the PDH E1-alpha and E3 subunit genes of *Plasmodium yoelii* can be disrupted with no obvious growth phenotype until the liver stages [55], given that GPI biosynthesis is essential for blood-stage survival [56]. It is possible that in the event of PDH inactivation a separate acetyl-CoA-generating mechanism (discussed below) might compensate, indicating a degree of metabolic flexibility within this pathway. Nonetheless, the importance of GPI-anchored proteins to parasite development and the unique nature of certain of the enzymatic steps have suggested this pathway as a potential drug target [54].

6. Lipid biogenesis

The highly proliferative growth of the blood-stage parasite depends on manufacturing significant quantities of new membrane. However, early experiments tracking the incorporation of radio-labeled glucose revealed that the amount of carbon from this nutrient incorporated into the lipid fraction of *P. knowlesi* biomass was low and occurred mainly in the glycerol backbone instead of the acyl chains [5]. This suggests that *de novo* fatty acid biosynthesis, or at least that using carbohydrates as a carbon source, is not a significant component of parasite metabolism. Interest in fatty acid metabolism has recently resurged with the discovery that the parasite genome encodes the complete suite of enzymes necessary for type II (bacterial) fatty acid synthesis (FAS). These enzymes are all targeted to the apicoplast, a nonphotosynthetic plastid-like organelle derived from a secondary endosymbiotic event that is suspected to play a role in a number of plant-derived metabolic pathways [25,57]. Type II FAS is an attractive drug target and con-

siderable excitement has been generated by the discovery that triclosan, an antibiotic targeting bacterial enoyl-acyl carrier protein reductase (ENR), efficiently inhibits both the incorporation of ^{14}C-acetate into parasite fatty acids and *P. falciparum* growth in culture [58]. However, the transcripts for various enzymes in the Type II FAS pathway show very low to undetectable blood-stage expression levels in microarray analyses [26], and subsequent investigations determined that several of these, including ENR, could be genetically disrupted in both *P. berghei* and *P. falciparum* without any discernible affect on blood-stage growth [59,60]. However, the FAS pathway is essential during the liver stages, as these mutants exhibit a block in liver cyst development. Therefore, these studies conclusively demonstrate that ENR is not the blood-stage target of triclosan. That triclosan remains lethal to these ENR knockout parasites, and in fact still inhibits acetate incorporation [58], imply that it targets another aspect of parasite metabolism, perhaps acyl chain extension.

A more complete understanding of the structure of parasite fatty acid metabolism derives from a series of experiments involving metabolic tracers and different lipid precursor supplementations [61]. *P. falciparum* is typically cultured in serum-free medium supplemented with lipid-rich albumin, which is required for growth due to the complex mixture of fatty acids it provides. By systematically testing different combinations of free fatty acids for their ability to support parasite growth, Mi-ichi et al. were able to determine six combinations of C_{14}, C_{16}, C_{18} fatty acids and several desaturation products thereof that enable continuous culture of *P. falciparum*. No single fatty acid was required in every combination because the parasite possesses a limited ability to modify exogenously supplied fatty acids as needed. Specifically, metabolic labeling experiments using radioactive fatty acid species determined that the parasite can elongate C_{14} to C_{16} and C_{16} to C_{18}, as well as desaturate acyl chains (primarily at the ω-9 position).

A model that arises from the data above is of a lipid metabolism in which preformed fatty acids are (1) scavenged from the host cell and serum, (2) subjected to a limited set of modifications by parasitic elongases and desaturases, and (3) incorporated into membrane glycerides with a glycerol backbone derived ultimately from glucose through the EMP pathway (Fig. 1). The two-carbon units (in the form of acetyl-CoA) necessary for elongation might be produced from glycolytic pyruvate in the apicoplast by the PDH complex localized to that compartment [62]. However, this enzyme is not essential during the blood stages [55]. It is possible that a glutamine-driven pathway (discussed below) could supply acetyl-CoA to lipid elongation, either under normal circumstances or else solely in the PDH mutant. This remains to be demonstrated however, since the few metabolic labeling experiments to robustly label acyl chains in parasite cultures have used very high levels of free ^{14}C-acetate (*P. knowlesi* [5], *P. falciparum* [58]). In these experiments, only a small fraction (∼15%) of the total radioactivity incorporated into lipids from ^{14}C-glucose in *P. knowlesi* was detected in the acyl chain [5]. In addition, we have recently used gas chromatography-mass spectrometry profiling of lipids in parasites fed either ^{13}C-glucose or ^{13}C-glutamine, but were unable to measure any ^{13}C incorporation into fatty acids [38]. Fatty acids acquired through scavenging from the extracellular environment evidently cannot serve as carbon sources themselves due to the absence from the genome of any of the β-oxidation enzymes necessary for the breakdown of acyl chains. Whole, preformed lipids might in theory be scavenged directly from the host cell membrane or host serum, but there is no evidence of lipid (as opposed to fatty acid) scavenging from the extracellular environment. Moreover, there is a net 6-fold increase in the phospholipid content of the infected erythrocyte [63], indicating significant amounts of phospholipid biosynthesis on the part of the parasite. The 'bewildering' series of plasmodial pathways responsible for assembling complex phospholipids, and the open questions pertaining to their study, have been very well reviewed elsewhere [64].

7. Tricarboxylic acid metabolism

Mitochondrial tricarboxylic acid (TCA) metabolism has long been considered a "black box" within the rest of the malaria parasite's metabolism, unclear both in terms of its function as well as its basic architecture. In most free-living microbes this pathway acts as a versatile central hub of carbon metabolism in which carbon derived from glycolysis or other nutrients (ethanol, acetate, amino acids, etc.) is fully oxidized to carbon dioxide while generating energy (by oxidative phosphorylation), reducing equivalents and biosynthetic precursors (such as for amino acid synthesis). However, from the earliest reports in the malaria literature onwards a more confusing and contradictory picture emerges. Electron micrographic observations of the human malaria parasites' morphology revealed the single mitochondrion to be only minimally cristate [65], calling into question its role as a site of active metabolism (although cristae are observed in the mitochondria of avian parasites [65,66]). The low levels of oxygen consumption observed in culture and the centrality of anaerobic glycolysis to energy metabolism discussed above further implied that the primary mitochondrial function of oxidative phosphorylation was absent or of minimal importance. In fact, the absence of obvious homologs to the F_0 *a* and *b* subunits of the F_1F_0 ATP synthase in the parasite genome led to speculation that this enzyme complex may be incapable of generating ATP [25], although candidate homologs have recently been identified bioinformatically [67]. Malaria parasites have also dispensed with *de novo* amino acid biosynthesis, instead acquiring them through scavenging from the host serum or hemoglobin catabolism [68], which generates such an excess of amino acids that most are excreted from the infected cell as waste [69]. Biochemical and informatic studies suggest that the parasite malate dehydrogenase is actually cytosolic [70,71], and so cannot contribute to a mitochondrial TCA cycle. Furthermore, as mentioned above the absence of the PDH enzyme complex from the mitochondrion [62], where it generally serves as the fundamental link between carbohydrate and mitochondrial carboxylic acid metabolism, converting glycolytic pyruvate to mitochondrial acetyl-CoA for citrate synthesis, raised the question of how, if at all, carbon enters the TCA cycle. This coincides with early reports that only very minimal amounts of $^{14}CO_2$ and keto acids are produced from ^{14}C-glucose during intraerythrocytic growth of both simian and avian parasites [1,2,9].

However, other lines of evidence indicate that some form of mitochondrial carboxylate metabolism occurs during the blood stage. The simian malaria parasite *P. knowlesi* and avian parasite *P. lophurae* fix $^{14}CO_2$ into metabolites including citrate, malate and succinate [8,72]. With the advent of the genome sequence it was realized that the parasite encodes homologs to all of the enzymes necessary for a complete TCA cycle [25], which are all are roughly coexpressed during the intraerythrocytic stage [26] and possess putative mitochondrial signal sequences, save for malate dehydrogenase. In place of malate dehydrogenase, a second enzyme capable of essentially running the same reaction (malate:quinone oxidoreductase, PFF0815w) is expressed and appears to be mitochondrially targeted. In addition, the *P. falciparum* citrate synthase homolog (PF10_0218) [73], aconitase (PF13_0229) [74] and isocitrate dehydrogenase (IDH, PF13_0242) [38] have been localized entirely or partially to the mitochondrion. Intriguingly, while most eukaryotes possess at least three isoforms of IDH (mitochondrial NADP-dependent, mitochondrial NAD-dependent and cytosolic NADP-dependent), the parasite encodes only the mitochondrial NADP-dependent enzyme. Enzymatic activity for the TCA cycle

enzymes succinate dehydrogenase (PFL0630w and PF10_0334) [75], aconitase [74] and IDH (in *P. knowlesi*) [76] have been detected in parasite extracts, and IDH has been cloned and characterized [77,78]. Furthermore, the *Plasmodium* spp. possess an essential *de novo* heme biosynthetic pathway which presumably requires succinyl-CoA to function [79]. As the only known source of this precursor in the parasite's metabolic network, at least a subset of the TCA cycle enzymes (2-oxoglutarate dehydrogenase or succinyl-CoA synthetase) must be active. Finally, a metabolomic survey of the *P. falciparum* blood-stage developmental cycle discovered that the levels of several TCA cycle intermediates (ketoglutarate, iso/citrate, aconitate) oscillate periodically over the intraerythrocytic developmental cycle roughly in phase with the TCA cycle enzymes, suggesting that they are actively synthesized [80]. In order to explain these disparate strands of data, several models have been put forward. One suggestion is that the full TCA pathway is active during different parasite life cycle stages, with the parasite's branched-chain alpha-keto-acid dehydrogenase complex possibly supplying mitochondrial acetyl-CoA through amino acid catabolism [81], or perhaps by acting as a surrogate pyruvate dehydrogenase [82].

Efforts to address this enigma in our laboratory have taken advantage of recent advances in mass spectrometry-based high-throughput chemimetric technologies [83–85]. These metabolomic platforms have the advantage of permitting rapid and simultaneous quantification of a large panel of cellular metabolites, and, using mass spectrometry for detection, allows for relatively simple metabolic tracing experiments using stable isotope-labeled nutrients in which the number and position of labeled atoms can be determined. By feeding *P. falciparum* cultures ^{13}C-glucose and analyzing isotopic distributions we have confirmed that carbohydrate metabolism does not contribute significantly to the TCA cycle, though there is clear evidence of cytosolic CO_2 fixation through PEP carboxylase to oxaloacetate and malate [38]. ^{13}C-glutamine, by contrast robustly labels all TCA cycle intermediates measured. It seems that glutamine is efficiently taken up by the infected cell [86] and converted to glutamate (likely through glutamate synthase, PF14_0334), which is itself converted to 2-oxoglutarate by glutamate dehydrogenase (GDH). *P. falciparum* encodes 3 distinct GDH genes: an NADP-dependent enzyme presumably targeted to the apicoplast (PF14_0286) [87], which may supply NADPH for biosynthetic reactions in that compartment; another NADP-dependent GDH lacking a cleavable signal sequence, and so presumably cytosolic (PF14_0164) [88]; and a large, fungal-type NAD-dependent GDH (PF08_0132) [87] with no predicted signal sequences. While we can only speculate at this point which of these predominates in terms of flux, one hypothesis that has been put forward has it that the cytosolic NADP–GDH might be a major source of NADPH for redox control [12], which would concomitantly generate cytosolic 2-oxoglutarate. Studies in our lab have determined that a significant amount of 2-oxoglutarate is effluxed from infected cells as an apparent waste product, but some fraction appears to enter the mitochondrion, possibly through a malate:oxoglutarate antiporter homolog (PF08_0031), and serves as the carbon source driving TCA metabolism [38].

Surprisingly, the observed isotope labeling patterns when cells are fed ^{13}C-glutamine indicated that this metabolism comprises two largely independent pathways: an oxidative branch running successively from 2-oxoglutarate to succinyl-CoA, succinate, fumarate and malate (essentially half of a canonical TCA cycle), and a reductive branch running from 2-oxoglutarate to isocitrate, citrate, oxaloacetate and malate (Fig. 1). We find that the resulting malate is excreted along with malate produced during the cytosolic carbon fixation process. The oxidative branch is unlikely to carry a very high flux but should generate reducing power (NADH) for the electron transport chain, GTP (via substrate-level phosphorylation) and, critically, the succinyl-CoA required for heme biosynthesis. The reductive branch, by contrast, produces two-carbon units during the citrate cleavage step rather than consuming two-carbon units as acetyl-CoA.

These results help to clarify a long-standing mystery in the field of *Plasmodium* metabolism by revealing a significantly diverged architecture for this fundamental pathway. Several other puzzling observations become explicable in the context of this model. For example, losing the canonical TCA cycle's NAD-dependent IDH while retaining the NADP-dependent isoform may be explained by noting that the reaction catalyzed by the NAD-dependent isoform is typically irreversible in the oxidative direction [89] while the NADP-dependent reaction is freely reversible in a wide variety of organisms [89–92]. Thus this permits the pathway to run in the reductive direction. Also, the presence of the putative mitochondrial malate:oxoglutarate antiporter has been difficult to interpret given the absence of any identifiable glutamate:aspartate antiporter, as the pair normally function together to complete the metabolic circuit known as the malate–aspartate shuttle [81]. However, if the mitochondrion is consuming 2-oxoglutarate and excreting malate then this transporter's role would simply be to exchange substrate for product. Other questions remain open: our data suggest that the parasite has evolved (at least) two independent pathways for the production of acetyl-CoA, and that these two pathways therefore might play different metabolic roles. However, it is not completely clear at this point whether this reductive pathway localizes entirely to the mitochondrion, or how essential it is for parasite growth. Further study is required to elucidate these and other puzzles.

8. Systems level studies

A variety of recent research efforts have aimed to elevate our understanding of plasmodial biology and metabolism to a systems level through the use of high-throughput "-omics" approaches, both in *in vitro* and *in vivo* settings. Though generally more difficult to interpret than classical approaches directed at one to a few nodes in a biological pathway, such datasets offer the potential to capture behaviors of the entire network. A study of the transcriptional profiles of *P. falciparum* samples harvested directly from clinical malaria patients for example, found that the parasites may exist in three broad clusters that seem to reflect a difference in metabolism [93]. Based on the gene expression of glycolytic and TCA cycle genes, the authors concluded that one cluster resembled normal anaerobic fermentative metabolism commonly seen in culture, while another seemed to show evidence of a starvation response and a switch to oxidative energy generation. Our biochemical evidence described above renders it doubtful that oxidative phosphorylation plays a significant energetic role, although it is possible that these data indicate an up-regulation of mitochondrial metabolism to generate two-carbon units in under conditions of reduced glucose availability in starved parasites (although other investigators have challenged these results as artifacts of analysis [94]). Several groups have reported that TCA cycle enzymes are present during all life cycle stages of the parasite analyzed, and may be particularly up-regulated in gametocytes, ookinetes and sporozoites of *P. falciparum* and *P. yoelii* [95–99]. The functional implications of this remain mysterious, given both the unlikely nature that this pathway plays an energetic role and the unclear metabolic requirements at the stages of development when the parasite is not actively proliferating.

Of course, metabolomic methodologies will be critical in the push towards dissecting the interlocking carbon metabolic pathways of the *Plasmodium* parasites and their host cells (reviewed

in [64,100]). Such techniques have been used to probe metabolic perturbations induced by malarial infection in *in vitro P. falciparum* cultures [80] and *in vivo* rodent malaria models [101,102]. More directly, the ability of analytic technologies such as mass spectrometry and nuclear magnetic resonance (NMR) spectroscopy to trace the flow of heavy isotope-labeled nutrients through pathways is proving to be exceptionally valuable in this regard. Our own pathway analysis of carbon flow in *P. falciparum*, discussed in detail above, resulted in a new model architecture for mitochondrial carboxylic acid metabolism [38]. An investigation of labeling from ^{13}C-glucose by NMR has suggested the intriguing possibility that, in addition to severely increasing the glucose consumption of the infected cell, *P. falciparum* is capable of somehow inhibiting glucose utilization by uninfected erythrocytes (seemingly through the inhibition of the host phosphofructokinase and pyruvate kinase by an excreted soluble factor) [33,103]. If this finding can be confirmed and the inhibitory molecule identified it would be a fascinating insight into the parasitic modulation of host metabolism. Another group has reported, again using NMR-based measurements of ^{13}C-labeled glucose metabolism, that *in vitro* cultures of *P. falciparum* produce glycerol as a major metabolite of glucose [104]. This finding is surprising given the rarity of glycerol production among eukaryotes and the absence of any enzyme in the current *P. falciparum* genome annotation capable of generating glycerol, such as a glycerol-3-phosphatase or a reversible glycerol kinase, although this activity is found in other parasitic protozoa such as the *Trypanosomes* [105–107]. These authors hypothesize that a functional glycerol-3-phosphate shuttle is therefore present in *Plasmodium* spp. and that a reappraisal of parasite redox metabolism may be necessary. However, since no quenching or deproteination step was used during metabolite extraction, it is possible that the observed glycerol may be the product of a nonspecific phosphatase acting on glycerol-3-phosphate produced by the parasite (for example, the excreted acid phosphatase discussed above was found to cleave phosphate from a wide variety of small-molecule substrates, including glycerol-2-phosphate [28]). Nevertheless, these recent observations suggest that the parasite may have some further metabolic tricks up its sleeve and that our current views of redox control and carbon metabolism should be interpreted with an open mind.

9. Suggested research avenues and concluding remarks

The preceding decades of malaria research have vastly clarified our understanding of the biology of the parasite and filled in many of the blank spaces of its metabolic map. The challenge now facing the malaria community is to build on the foundation of these results a comprehensive understanding of *Plasmodium* physiology, metabolism and host-parasite interactions both at the cellular and organismal level. One major stumbling block on the way toward this goal is our poor understanding of the metabolic interconnection between the RBC and its invader. The infected cell is highly compartmentalized and in one sense the essence of intracellular parasitism is the exchange of nutrients and wastes between parasite, host cell and environment. This is due largely to the significant difficulty inherent to compartmental analysis of metabolite concentrations and fluxes and a paucity of information about the localization and even identity of plasmodial proteins (most of which are unannotated). One clear starting point to unraveling this web of interactions is a concerted effort to identify and localize the many putative transporters of unknown function expressed during the blood stage [108]. A more complete understanding of the transport capabilities of the infected cell will significantly aid in efforts to understand how *Plasmodium* spp. mediate their exchange with the environment through the host cytoplasm.

Another fruitful area of inquiry is the genetic control of metabolism. Many regulatory interactions are difficult to study in malarial systems due to the lack of genetic tools. However, the progeny of phenotypically distinct strains have been used in conjunction with quantitative trait loci (QTL) analysis to uncover the loci governing traits such as drug resistance [109] and mRNA expression level [110]. This tool has also been used with metabolomics to probe metabolic regulation in *Arabidopsis thaliana* [111,112]. Such an approach has enormous potential in malaria research to reveal interactions not accessible by other methods.

A more fine-scaled understanding of not just the architecture of the metabolic pathways of the infected cell but their dynamics is achievable through the use of fluxomics to map out the fluxes through the metabolic network. Such studies entail using a variation on classical pulse-chase techniques, rather using stable isotope-labeled nutrients and analytical platforms (MS or NMR-based) capable of measuring changes in the isotopic labeling pattern of downstream intermediates. Such studies measure both steady-state fluxes and the transient change in fluxes induced by a perturbation, both of which provide significant insight into the dynamic regulation and enzyme kinetics at play in the system [113–115]. Again, several technical challenges must be resolved before such studies can be carried out with current *in vitro* malaria culture techniques, which are complicated by the presence of host cell compartments, enzymes, the presence of uninfected cells and other, more subtle peculiarities of the system. For example, our initial investigations into the TCA cycle were complicated by the observation that citrate concentrations in our RBC extracts were far in excess of the negligible erythrocyte citrate content reported in the literature [116]. We determined that this was due to citrate entering the RBCs during storage in the high citrate (>10 mM final concentration) anticoagulant solutions routinely used during blood collection. This issue was easily resolved using blood collected in sodium heparin solutions [38] but points to the special care that must be taken to minimize the effect of metabolic perturbations resulting from the nutrient environment during *in vitro* tissue culturing.

The avenues above will ultimately prove their worth by contributing to a systems level model for *Plasmodium* metabolism. Flux-balanced (stoichiometric) *in silico* metabolic networks have been developed for well-studied model organisms such as yeast [117] and *Escherichia coli* [118] as well as several pathogens [119–121], and recent efforts by several groups have focused on constructing such an informatic tool for *P. falciparum* [122–124]. At present these are limited by our lack of understanding about the structure, dynamics and compartmentalization of the infected cell's metabolism, and such models must be refined by experimental evidence such as described above. A sufficiently accurate model will permit immediate *in silico* experiments to determine vulnerable drug targets (enzymes and transporters) and multi-drug dosing strategies, potentially significantly speeding the process of rational drug design. A fundamental understanding of central carbon metabolism in *Plasmodium* spp., and the unique adaptations differentiating the parasite and its host provide exciting new avenues for future therapeutic intervention strategies.

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