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Multiple roles of proline transport and metabolism in trypanosomatids

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1. ABSTRACT

Trypanosomatids are a large family of unicellular eukaryotes, many of which are parasites in higher eukaryotes including man. Much of our understanding of metabolism in these organisms has been gained form the study of the human infective representatives (*Trypanosoma brucei* subpecies, *Trypanosoma cruzi* and *Leishmania* spp.) which are transmitted by blood-feeding arthropods. The insect vectors of these parasites use proline as a principal carbon and energy source circulating in their haemolymph. Accordingly the insect-forms of the human infectious parasites have evolved to exploit abundant proline when in this environment, but being able to activate different biochemical pathways when in other environments. Interestingly, if glucose is available, metabolic capability can shift to make this carbohydrate the preferred substrate. Proline has also been shown to play key roles in osmoregulation, differentiation in representatives of the group and may even play a role in immunosuppression elicited by the American trypanosome *T. cruzi*. This review focuses on recent progress in understanding the different aspects of proline metabolism in trypanosomatids, with a particular interest on the insect forms.

2. INTRODUCTION

Trypanosomatids are eukaryotic parasitic protozoans belonging to the order Kinetoplastida. Around 24 million people are infected with trypanosomatids worldwide, with over 600 million people at risk from diseases caused by these parasites, such as sleeping sickness (causative agent: *Trypanosoma brucei* spp. - African trypanosomes), Chagas disease (*Trypanosoma cruzi*) and leishmaniasis (*Leishmania* spp.). Currently, there are no vaccines and treatments are relatively dangerous and ineffective even though the human diseases can be highly disabling and even fatal. For example, sleeping sickness is fatal if untreated, but existing medications have severe side effects (including death) and drug resistance is increasing. The situation is similar for Chagas disease and leishmaniasis. Since trypanosomiasis and leishmaniasis afflict predominantly people from the world's poorest populations, economic incentives to invest in interventions are limited and thus these are considered 'neglected diseases'. Other trypanosomatid species cause diseases affecting domestic animals (*e.g.* the African trypanosomes: *T. congolense*, *T. vivax*, *T. evansi* etc. and *leishmania* spp.) and contribute significantly to the economic burden of developing economies.

The cell biology of trypanosomatids is extraordinary. These organisms are characterized by several unique processes that have been the subject of extensive research, such as atypical gene expression (1), mRNA maturation by trans-splicing (2), editing of mitochondrial RNA (3), antigenic variation (4), localisation of glycolysis in glycosomes (peroxisome-like organelles) (5), and fatty acid biosynthesis that is performed by elongases instead of the canonical type I or type II synthetases (6). These unusual biological, genetic and biochemical features have stimulated broad scientific and evolutionary studies making trypanosomes popular model organisms. The African trypanosome is amenable to genetic manipulation through gene knockout and RNA interference (RNAi), the latter a very powerful reverse genetic tool that allows selective ablation of gene expression (7). RNAi is also operational in *Leishmania* subgenus *Viannia* (8), but not in other *Leishmania* spp. (9) or in *T. cruzi* (10). Notably, the emerging availability of genome sequences is expected to facilitate insights into these organisms, for example it has exposed the metabolic potential of these cells (11, 12). To date, genome sequencing of *T. brucei* (11), *T. cruzi* (13) and three *Leishmania* species (1, 14) has been undertaken and others projects are underway.

The purpose of this article is to review proline utilization in trypanosomatids, focusing on its transport, its metabolism for energy supply and the roles of this amino acid in differentiation and osmoregulation.

3. DESCRIPTION OF TRYPANOSOMATIDS

3.1. *Trypanosoma brucei*

Subspecies of *Trypanosoma brucei* are responsible for human African trypanosomiasis (HAT). This disease is also referred to as sleeping sickness since after the parasites have left the bloodstream (stage 1 disease), they become established within the central nervous system (stage 2 disease) and cause, among other neurological sequelae, a breakdown in sleep-wake patterns (15, 16). *Trypanosoma brucei gambiense*, responsible for over 90% of all HAT cases, is found in west and central Africa whereas the other key causative agent, *T. b. rhodesiense* is found in eastern and southern Africa (17). Generally, in the case of gambiense disease, there are several years between infection and death; death occurring during the neurological stage, whilst a more rapid disease course is typical after rhodesiense infection. African trypanosomes employ a well-studied process of antigenic variation to avoid detection by mammalian immune systems (18). In addition, subspecies that infect humans are resistant to a lytic factor comprising apolipoprotein L1 and a haptoglobin-like protein that is present within the high density lipoprotein fraction of blood, whereas other subspecies (*T. b. brucei*) and related species that infect various other mammals are sensitive to this factor (19, 20).

The prevalence of sleeping sickness was nearly 500,000 cases at the end of the twentieth century but targeted intervention, based on chemotherapy and control of the tsetse fly that transmits the disease, has recently brought incidence down to fewer than 10,000 cases. The type of chemotherapy given for sleeping sickness depends on whether *T. b. gambiense* or *T. b. rhodesiense* is the infectious agent and also on the disease stage (21). Pentamidine and suramin are usually administered to patients suffering from stage 1 gambiense and rhodesiense disease, respectively. For stage 2, melarsoprol is the only drug in use for rhodesiense disease and eflornithine, either as monotherapy or preferably in combination with nifurtimox, is the treatment for gambiense disease. Each drug has associated drawbacks, such as toxicity, a need for potentially protracted parenteral administration, cost and distribution issues. The need for new therapies has driven interest in learning more about the biochemistry of these parasites (22).

The *T. brucei* life cycle is complex; the parasite must adapt to life in both its insect (tsetse fly) and mammalian hosts (Figure 1). The biochemistry of the African trypanosome reflects adaptation to these dissimilar environments (23). For example, in the mammalian bloodstream the parasites depend only on catabolism of glucose, which is abundant in blood, and the predominant end product of this catabolism is pyruvate. The first seven steps of the glycolytic pathway reside within a peroxisome-like organelle, the glycosome (24), an arrangement that allows for rapid consumption of glucose and unique regulatory mechanisms different from those employed in other cell types (25). Mitochondrial metabolism is downregulated in bloodstream forms of the parasite. In contrast, in the tsetse fly glucose is generally not abundant (other than directly following feeding) and L-proline is the principle carbon and energy source. Accordingly, the trypanosome forms in this environment, the most extensively studied being the cultured procyclic midgut stage parasite, consume L-proline (26, 27). Additionally, the mitochondria of procyclics exhibit altered morphology

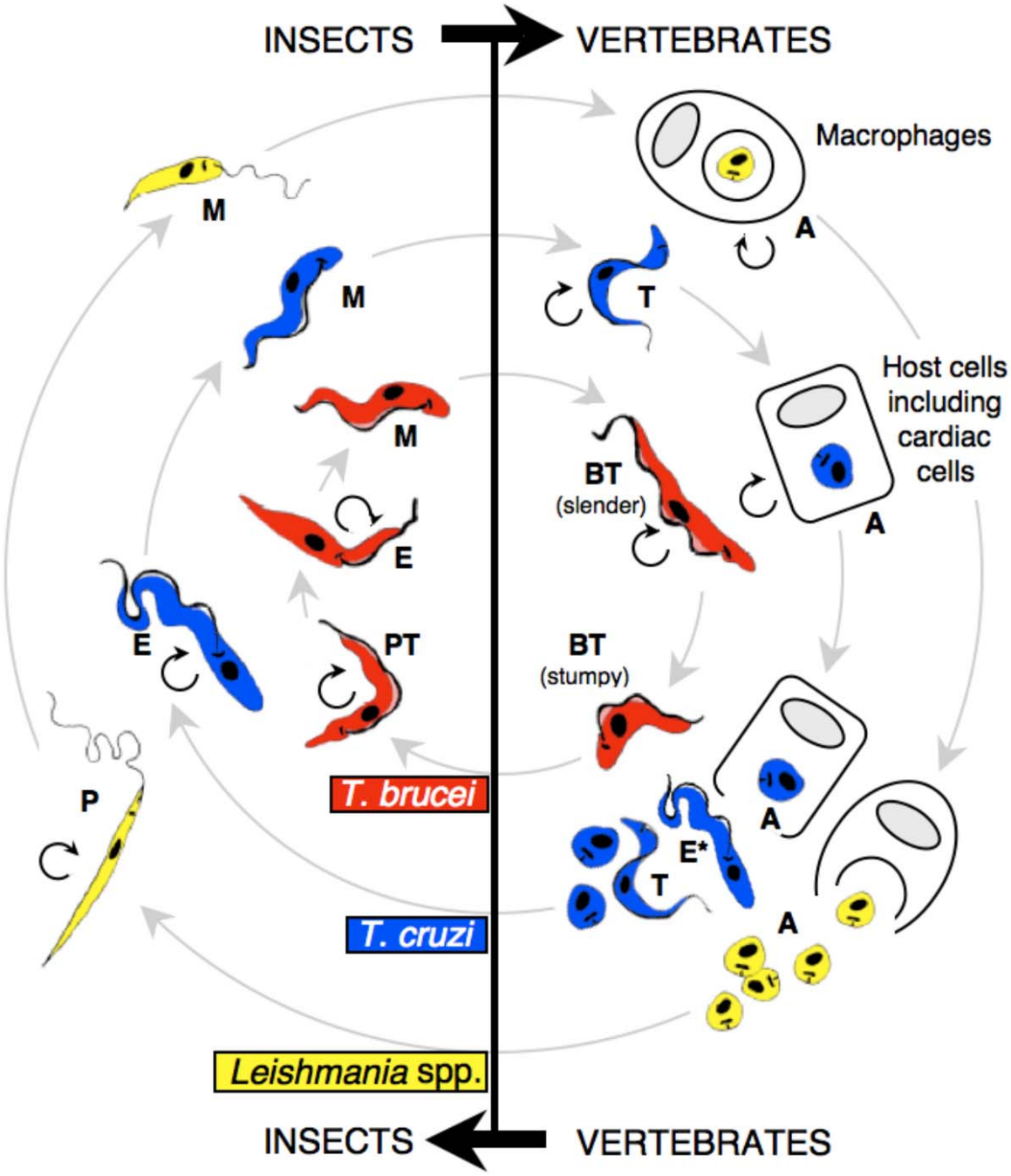

Figure 1. Life cycle of human infective trypanosomatids (adapted from (12)). Circular arrows designate duplicative forms. Abbreviations: A, amastigote; BT, bloodstream trypomastigote; E, epimastigote; M, metacyclic; P, promastigote; PT, procyclic trypomastigote; T, trypomastigote.

and physiological status, becoming cristate and expressing enzymes responsible for tricarboxylic acid (TCA) metabolism (albeit without a classically functioning Krebs cycle (28)) and the respiratory chain.

3.2. *Trypanosoma cruzi*

Trypanosoma cruzi is the causative agent of Chagas disease (15, 29). Currently clinical classification of Chagas disease is being re-evaluated since there are at least six clearly genetically distinct causative types (TcI-TcVI) (30) with variability in associated symptoms. Up to 8 million individuals are presently infected with *T. cruzi* (29). The natural transmission cycle is restricted to Latin America where the triatomine bugs responsible for transmission are found (see Figure 1 for the life cycle), but the migration of hundreds of thousands of people, including

up to 300,000 that have re-located to the USA (31), has caused Chagas disease to become an international problem. The parasites are transmitted in the feces of bugs feeding on mammalian hosts. Bloodstream form trypomastigotes enter the host and eventually invade multiple different cell types, therein transforming into cytoplasmic amastigote forms that replicate. Amastigotes can undergo long periods of apparently reduced activity in the form of pseudocysts. Consequently, after the initial infection, patients can enter a long, so-called indeterminate phase when it is difficult to identify parasites (32). These persistent forms appear to trigger a progressive inflammatory host response over the succeeding years (33). Death can occur up to twenty years after infection and is associated most frequently with inflammatory lesions in the heart, colon or esophagus (34). The prevalence of Chagas disease has been reduced greatly, particularly in the southern cone countries, by significant interventions against the insect vectors (35). The only registered treatments for Chagas disease are two nitroheterocyle drugs (nifurtimox and benznidazole); these are curative in the early stage but ineffective in the chronic phase (36). Consequently, there is an urgent need for new therapies.

Many of the biochemical characteristics of other trypanosomatids are also features of *Trypanosoma cruzi* (37). However, because these parasites replicate in the mammalian host as intracellular amastigotes they confront a metabolic environment different to that encountered by bloodstream form African trypanosomes; for example free glucose is greatly depleted intracellularly. Accordingly, the mammalian and insect forms of *T. cruzi* are more similar metabolically, the mitochondrion active in both life cycle stages.

3.3. *Leishmania spp.*

Protozoan parasites of the genus *Leishmania* are the causative agents of a wide spectrum of human and animal diseases. Infections with the numerous different species of *Leishmania* are associated with diverse clinical manifestations, ranging from lesions of the skin (cutaneous leishmaniasis) and mucous membranes (mucocutaneous leishmaniasis) to visceral and ultimately lethal lesions (kala azar) (38). *Leishmania* species cause morbidity and mortality across 66 countries in the Old World and 22 in the New World. It is estimated that over 12 million people are afflicted with leishmaniasis worldwide, some with overt disease and others with no apparent symptoms. Of the 1.5-2 million new cases of leishmaniasis estimated to occur annually, only 600,000 are officially declared. AIDS and other immunosuppressive conditions increase the risk of *Leishmania* infected people developing visceral illness. In certain areas of the world, such as south-west Europe, South America, Asia and Africa, the risk of co-infection with HIV is rising due to epidemiological changes (39, 40). For many years pentavalent antimonials have been used to treat leishmaniasis, but other compounds are now available such as amphotericin B, paromomycin and oral miltefosine. The choice of drug and dosing regimen are species specific (41).

Leishmania cycle between phagolysosomes of mammalian macrophages and the alimentary tract of female sand flies (Figure 1), growing as flagellated extracellular promastigotes in the vector and as aflagellated intracellular amastigotes in the host. During this life cycle, the environment of the organisms changes from relatively alkaline, sugar- and amino acid-rich, to acidic, fatty acid- and amino acid-rich (42-44). More specifically, promastigotes are introduced into the host during a vector's blood meal and are subsequently phagocytosed by macrophages, where they differentiate into amastigotes (45, 46). This developmental process can be triggered *in vitro* by shifting cultured promastigotes (grown at 26ºC, pH 7) to a lysosome-like environment (37ºC and pH 5.5), a protocol used widely to study mechanisms of differentiation (47-49).

4. PROLINE TRANSPORT AND ACCUMULATION

4.1. Proline transport in *Trypanosoma brucei*

L'Hostis *et al.* carried out kinetic analysis of L-proline transport in procyclic *T. brucei* using a rapid centrifugation through oil approach (50). Uptake of L-proline was ascertained to be linear over the first 35 sec. Uptake over a range of L-proline concentrations indicated a transporter with a K_m value of 18.7 μM and a V_{max} value of 16.1 nmol·min^{-1}·10^{8} cells^{-1}. The shape of the Michaelis-Menten curve was consistent with the presence of a single transporter. A simple competitor analysis, where uptake of 0.1 mM labelled L-proline was assayed in the presence of 1 mM potential competitors, revealed that of the other 19 common proteinogenic amino acids only L-alanine (51% inhibition) and L-cysteine (35% inhibition) competed, implicating these amino acids as probable co-substrates for the same transporter (all other amino acids inhibited by <15%). Hydroxyproline (66% inhibition) and L-azetidine-2-carboxylic acid (46% inhibition) also compete with L-proline uptake. Notably, L-proline uptake was observed to occur against its concentration gradient such that there is significant accumulation inside procyclic *T. brucei* (up to 270-fold with a 20 μM external concentration), suggesting that an energy source drives active transport. However, since extracellular pH changes were not associated with altered uptake and the protonophore, CCCP, did not inhibit uptake, it appears a proton motif force does not drive transport. Furthermore, replacing Na^+ ions with Li^+ had minimal effect on L-proline uptake and ionophores, including monensin and valinomycin, did not inhibit uptake. Nevertheless, KCN, which inhibits ATP production *via* the respiratory chain, was found to inhibit L-proline uptake and in reconstituted membrane systems intracellular ATP is required for L-proline permeation. These findings support that L-proline uptake in procyclic *T. brucei* is active but uses an unidentified motive force.

L-proline uptake has also been measured in another procyclic trypanosome strain (strain 427) (26). In accord with the study of L'Hostis *et al.*, kinetic analysis pointed to a single transporter with an apparent K_m for L-proline of 21 μM and a V_{max} value of 0.7 nmol·min^{-1}·10^{8} parasites^{-1} when cells were grown in SDM79 medium. Notably, the V_{max} was observed to increase to 18 nmol·min^{-1}·10^{8} parasites^{-1} in cells cultivated in glucose-depleted medium indicating that substrate availability regulates uptake. Taken together, the two studies establish that L-proline uptake into procyclic *T. brucei* is mediated by a

single transporter with a K_m of around 19 μM and capacity to transport against a concentration gradient. The transporter is relatively specific for L-proline, though L-alanine and L-cysteine are possible substrates. L'Hostis *et al.* also assayed L-proline uptake in *T. brucei* bloodstream forms and found it reduced relative to procyclic forms.

A gene (or genes) encoding an L-proline transporter(s) has not been identified yet in *T. brucei*. Based on the classification proposed by Jackson (51), data from the genome sequencing project suggest that the *T. brucei* genome contains 17 different loci containing amino acid transporter related genes (TbAAT1-TbAAT17). Divergent transporter sequences are found clustered at a number of these loci and thus it seems that there are around 46 distinct genes encoding amino acid transporter family members in *T. brucei*. To date no studies have dissected individual gene functions by expressing genes heterologously. A recent report proposed that TbAAT6 encodes a transporter for the amino acid analogue eflornithine (difluormethylornithine; DFMO) as this transporter is lost in trypanosomes resistant to the drug (52) and TbAAT6 knockdown by RNA interference renders trypanosomes resistant to the drug (52-54). Furthermore, uptake of eflornithine into trypanosomes lacking TbAAT6 is greatly reduced. In the absence of functional information it is not possible to say which of the ~46 TbAAT genes (or possibly other genes) encode the *T. brucei* L-proline transporter. However, as genes encoding L-proline transporters are identified in other trypanosomatids, it is likely that syntenic gene(s) will prove responsible for L-proline uptake in *T. brucei* (see section 4.3).

4.2. Proline transport in *Trypanosoma cruzi*

Silber *et al.* performed kinetic analysis of L-proline uptake in *T. cruzi* epimastigotes (55). When uptake is measured over a 30 second time period, the shape of the Michaelis-Menten curve is biphasic indicative of two transport systems, termed A and B, with K_m values of 310 μM and 1360 μM and V_{max} values of 12 pmol·min^{-1}·2x10^7 cells^{-1} and 65 pmol·min^{-1}·2x10^7 cells^{-1}, respectively. Competition analysis indicate that system B is specific for L-proline, L-alanine and L-cysteine whereas system A transports, in addition to these three amino acids, L-methionine, L-tryptophan, L-leucine and L-glycine. Both system A and B appear to be energy dependent as oligomycin, rotenone and antimycin A inhibit uptake in each case; however, Silber *et al.* concluded that system A is H^+ dependent whereas system B uses an unknown motive force.

4.3. Proline transport in *Leishmania spp.*

Anthony Mukkada, who pioneered solute transport research in *Leishmania*, was the first to report active accumulation of L-proline in *L. major* promastigotes through a carrier-mediated system (56). However, the specificity of the transporter appeared to be broad, encompassing amino acids such as L-alanine, L-methionine, L-valine and various L-proline analogs. Subsequently, Bonay and Cohen proposed that L-proline uptake in promastigotes is mediated by one arm of a neutral amino acid transport system that comprises two neutral amino acid transporters, one for L-proline and the other for L-alanine, both with similar kinetics (57). Indeed, several years later Zilberstein and colleagues delineated that promastigotes have two distinct transport systems that translocate neutral amino acids; system A, a cation-dependent, broad specificity L-proline/L-alanine transporter, and system B, a cation-independent L-proline-specific transporter (58; E. Inbar, D. Zilberstein *et al.*, unpublished data).

L-Proline is accumulated actively by promastigotes and amastigotes against its concentration gradient (58-60). A proton electrochemical gradient across the parasite's plasma membrane drives L-proline transport, using symport with protons (59-62). This notwithstanding, *L. donovani* promastigotes grown in a chemostat under continuous glucose-free conditions with varying concentrations of L-proline were observed to adapt cellular L-proline levels to reflect those in the growth medium, indicating that under such conditions L-proline transport is not active (63).

Kinetic analyses uncovered differences between L-proline transport in *L. major* and *L. donovani* promastigotes. The K_m for L-proline in *L. major* is 60 μM and in *L. donovani* is 650 μM (56, 57, 61; E. Inbar, D. Zilberstein *et al.*, unpublished data). However, the K_m for L-proline is 48 μM in *L. donovani* promastigotes adapted to growth in a chemostat (63). The rate of transport is also dissimilar between the two species, 64 *versus* 12 nmoles·mg protein^{-1}·min^{-1} in *L. donovani* and *L. major*, respectively (56, 61; E. Inbar, D. Zilberstein *et al.*, unpublished data). Notably, the *L. donovani* rate of L-proline transport resembles that of glucose transport in bloodstream *T. brucei* (64).

Amastigotes derived from hamsters accumulate L-proline at a lower rate and to a lesser extent than promastigotes (58, 60). Furthermore, the pH at which optimal transport occurs is dissimilar, pH 5.5 in amastigotes *versus* pH 7 in promastigotes. This lower pH for optimal L-proline uptake in amastigotes reflects the more general properties of L-proline metabolism in amastigotes; incorporation and oxidation were observed to be optimal when cells are cultivated in medium of pH 5 (65). In line with these differences between promastigotes and amastigotes, rapid acidification of the transport assay medium from 7 to 5.5 enhances uptake in amastigotes and suppresses it in promastigotes. Conversely, rapid alkalinification from 5.5 to 7 increases the rate of L-proline transport in promastigotes and suppresses it in amastigotes (60). Based on these observations, it was proposed that promastigotes and amastigotes possess different L-proline transporters. Indeed, a few years later, using an axenic system Mazareb *et al.* showed that amastigotes and promastigotes express distinct, stage-specific L-proline transporters (58).

Leishmania promastigotes differentiate into amastigotes straight after moving from the sand fly (pH 7.5 and 26°C) into phagolysosomes (pH 5.5 and 37°C), a developmental process that involves retooling biochemical

pathways to survive the hostile environment of lysosomes (42, 66, 67). Accordingly, it was shown both *in vivo* and *in vitro* that not only pH but also temperature changes strongly affect L-proline transport activity (58, 60). The pH-dependence of L-proline transport supports the hypothesis that *Leishmania* parasites possess a sensing mechanism that enables rapid adaptation to changing pH as they move between vector and host (68). Accordingly, reducing the pH of the growth medium from 7 to 4.5 results in a dramatic decrease in both the rate and steady state level of L-proline transport in promastigotes; at pH 4.5 the V_{max} was one-tenth and the affinity for L-proline twice that at pH 7 (61). Furthermore, although the optimal pH for L-proline transport is 7 for promastigotes grown at pH 7, it decreases as the culture pH is lowered such that the optimal pH for transport is 5.5 for cells grown at pH 4.5. The steady state level of transport also decreases with growth medium pH; at pH 4.5 it is at least three times lower than at pH 7. Notably, these phenotypic changes in L-proline transport triggered by reduced pH are reversible, as switching the pH of the growth medium from 4.5 to 7 was found to revert L-proline transport back to the phenotype characteristic of cells grown at pH 7; this reversal takes less than 24 hours and requires protein synthesis (61). It is notable that the phenotype of L-proline transport in promastigotes at low pH resembles that described in amastigotes (60).

Taken together, the data indicate that L-proline uptake in *Leishmania* is carried out by three systems, (*i*) a promastigote-specific, cation-dependent, broad specificity system A that translocates L-alanine and L-proline, (*ii*) a promastigote-specific, cation-independent, L-proline specific system B and (*iii*) an amastigote-specific, cation-independent, broad specificity system C. Recently, the gene encoding for system A was identified, cloned and characterized (E. Inbar, D. Zilberstein *et al.*, unpublished data).

4.4. Roles of proline transport in amino acid homeostasis and osmotic stress response

All living cells maintain a cellular pool of amino acids that provides precursors for metabolism and osmoregulation. The composition of this pool varies among organisms; the major components are glutamine, glutamate and threonine in mammalian cells (69), arginine, alanine and glutamate in the yeast *Saccharomyces cerevisiae* (70), and alanine, glutamate and proline in *Leishmania* and trypanosomes (71-73). Studies in bacteria, plant and mammalian cells indicate a critical role for amino acid transporters in determining the size and composition of these pools. To date the following transporters have been implicated in regulating the amino acid pool: ProP in *Escherichia coli*, SNAT2 in mammalian fibroblasts (69), Lht1 in *Arabidopsis thaliana*, and notably, LdAAP24 in *Leishmania* (E. Inbar, D. Zilberstein *et al.*, unpublished data).

Proline is uniquely abundant in the pool of trypanosomatids (~10 mM), a phenomenon that likely reflects adaptation of these parasites to the high proline level in the hemolymph of insect vectors. For example, the concentration of proline is 60 mM in the hemolymph of tsetse flies (*Glossina* species), the vectors of African trypanosomes (74) and is one of the most abundant free amino acids present in the hemolymph of *Glossina morsitans* fed on goats (75). In these as well as most other insects, the energy for flight is provided largely by oxidation of L-proline to L-alanine (76, 77). As might be expected given the prominence of proline in the trypanosomatid amino acid pool, it is well established that the insect forms of all trypanosomatids utilize L-proline as a carbon source (26, 27, 78-80) (and see below).

An important role of the trypanosomatid amino acid pool is to serve as a source for osmolytes during osmotic stress. In response to hypotonic stress *Leishmania* and trypanosomes activate rapid and massive amino acid efflux to minimize swelling (71, 72, 81). Indeed, both *L. major* promastigotes and *T. cruzi* epimastigotes have been shown to release amino acids from their pools within two minutes of a 2-fold hypotonic shift to reduce cellular osmolarity. The rate and extent of this release is proportional to the size of the hypotonic shift (71). Although many amino acids are released from the pool upon hypotonic shift, the key players vary among species. For example, only alanine and proline really influence hypotonic stress responses in *T. cruzi* (releases of 50 and 37 nmoles·mg cell protein^{-1}, respectively) whereas in *L. major* significant quantities of alanine (78 nmole·mg cell protein^{-1}), glycine, glutamate and serine (~25 nmole·mg cell protein^{-1}, each) are released. In *L. major* promastigotes the amount of proline released after a 2-fold hypotonic shift is only 14% that of alanine, suggesting that it has no role in hypotonic stress responses. Vieira *et al.* differentiated between two mechanistic features of osmotic stress responses, extent of swelling and rate of volume recovery (regulatory volume decrease, RVD) and showed that each is influenced by the cellular amino acid pool and cations in *Leishmania* (71). Specifically, promastigotes activate an amino acid channel that enables rapid amino acid extrusion to minimize cell swelling and subsequently recover cell volume by activating a regulatory volume decrease (RVD) mechanism (71, 81). Unlike in *Leishmania*, RVD appears to be less dependent on amino acid-independent in trypanosomes and involves water transport through aquaporin 1 in contractile vacuoles (82). However, in both *Leishmania* and trypanosomes, RVD requires cAMP-mediated signal transduction and phosphatidylinositol 3 (PI3) kinase activity (83, 84). Accordingly, inhibiting *T. cruzi* PI3 kinase homolog TcVps34 results in reduced RVD after hypotonic stress.

5. PROLINE METABOLISM

5.1. Introduction

The mammalian forms of trypanosomes and *Leishmania* consume primarily glucose as this is abundant in mammal fluids and consequently do not use L-proline as a carbon source. Similarly, mitochondria of the plant parasite *Phytomonas* isolated from *Euplorbia characias* are incapable of oxidizing L-proline (85). In contrast, the insect forms of trypanosomatids, such as the *T. brucei* and *T. congolense* procyclics, the *T. cruzi* epimastigote, *Leishmania* spp. promastigotes and *Crithidia* spp.

choanomastigotes, utlilize amino acids present in their vector host for energy production (80). As mentioned, L-proline is a predominant constituent of vector hemolymph and tissue fluids (74) and it has become clear that L-proline serves as a key carbon source for insect forms of trypanosomatids. Notably, field-isolated parasites have been adapted to *in vitro* axenic culture in glucose-rich media exemplified by the commonly used SDM79 medium, which contains this sugar at 6 mM. In these growth conditions, these insect parasites preferentially consume glucose over L-proline (26, 80, 86). As a consequence most attention has been focused on the analysis of glucose metabolism in these parasites. The significance of L-proline metabolism in insect trypanosomatid forms was only recently addressed after the development of glucose-depleted media (26, 28, 87, 88).

Several lines of evidence corroborate the essential role of L-proline metabolism in energy production in insect forms of trypanosomatids. Firstly, L-proline is essential for growth of the *T. brucei* procyclic EATRO1125 strain, regardless of the amount of glucose present in the medium (C. Ebikeme and F. Bringaud, unpublished data). Secondly, procyclic forms of several *T. brucei* strains can grow successfully in glucose-depleted medium, with no significant effect on growth rate (26, 87, 88). The same observation was made for the promastigote form of *L. mexicana* (89). Furthermore, knocking out all the glucose transporter genes from the *L. mexicana* genome does not compromise growth of the promastigote form (89). Thirdly, in the absence of glucose L-proline is the only amino acid capable of sustaining the growth of procyclic *T. brucei* and promastigote *L. donovani* (26, 63). Fourthly, the rate of L-proline consumption increases approximately 6-fold in insect forms of two different *T. brucei* strains in glucose-depleted medium. The latter finding not only highlights the key role of L-proline under these conditions but also indicates that glucose negatively regulates L-proline metabolism (26, 27, 90). The overall preference for glucose exhibited by insect trypanosomatid forms in rich medium likely reflects a preadaptation to deal with the high glucose concentration that will be encountered in the blood upon injection into the mammalian hosts.

Due to the initially under-appreciated role of L-proline metabolism, previous reviews concerning energy metabolism in insect trypanosomatid forms focused principally on glucose metabolism (23, 80, 91-97). Given the current understanding that L-proline is the primary carbon source for insect parasite forms, in the present review we describe in detail L-proline metabolism in trypanosomatids. Since this process is most studied in *T. brucei*, much of the data discussed pertain to that parasite.

5.2. Production of glutamate from proline

Like in all organisms, in trypanosomatids catabolism of L-proline is achieved by oxidation to L-glutamate. Specifically, production of L-glutamate from L-proline has been documented in the insect forms of *L. donovani* (98), *T. cruzi* (99), *T. congolense* (100) and *T. brucei* (27, 28, 101). Conversion of L-proline into L-glutamate involves two enzymatic steps (Figure 2). In the first step L-proline is oxidized to Δ-pyrroline-5-carboxylate (P5C) by flavoenzyme proline dehydrogenase (PRODH). Then P5C is hydrolyzed non-enzymatically to glutamic semialdehyde (γSAG), which is in turn oxidized to L-glutamate in the second step by NAD-dependent P5C dehydrogenase (P5CDH). Collectively, PRODH and P5CDH facilitate 4-electron oxidation of L-proline to L-glutamate. In most bacteria, the activities of PRODH and P5CDH are combined within a single polypeptide termed L-proline utilization A (PutA) flavoprotein (102). PutA proteins are peripheral membrane-associated enzymes when FAD is in its reduced state ($FADH_2$). However, when FAD is in its oxidized state, PutA proteins repress their own gene expression, as documented for the *E. coli* and *Salmonella typhimurium* enzymes (103). In contrast, in trypanosomatids, as in all eukaryotes and some prokaryotes, PRODH and P5CDH are distinct enzymes encoded by two separate genes (102) and PRODH is membrane-associated regardless of the oxidation state of the FAD prosthetic group. Yet, unlike the genomes of higher eukaryotes that can contain several *PRODH* genes (104, 105), a single *PRODH* gene is present in the trypanosomatid genomes sequenced to date (26).

Many organisms are able to perform the reverse process and synthesize L-proline from L-glutamate or ornithine (102). Irrespective of the starting material, γSAG and P5C are produced and the latter is converted into L-proline by a P5C reductase, which is encoded by all the trypanosomatid genomes currently sequenced (Figure 2). Notably, conversion of ornithine into γSAG is catalysed by the pyridoxal-5-phosphate-dependent enzyme ornithine aminotransferase (OAT), which is absent in trypanosomatids. The conversion of L-glutamate into γSAG involves two enzymatic activities, glutamate γ-semialdehyde and γ-glutamyl phosphate reductase. In plants and animals these two activities appear within a single bifunctional enzyme, P5C synthase, whereas in unicellular organisms, such as prokaryotes and yeast, the two activities are distinct enzymes. Genome sequences indicate that *Leishmania* spp. encode two separate enzymes, while *T. cruzi* encodes only the second enzyme and *T. brucei* lacks both genes (see: http://tritrypdb.org/tritrypdb/showRecord.do?name=GeneRecordClasses.GeneRecordClass&source_id=LmjF32.3140&project_id=TriTrypDB). L-proline biosynthesis has not been investigated directly in trypanosomatids and it remains unclear if and why this biosynthetic pathway is missing in trypanosomes but present in *Leishmania* spp.

It is noteworthy that OAT catalyses a reversible reaction that can be involved in ornithine production from L-proline to feed polyamine production, as previously described in the developing porcine placenta (106), or through the urea cycle. However, OAT and two key enzymes of the cycle are missing (ornithine carbamyltransferase and arginosuccinate lyase) (11) and experiments using ^{13}C labelled L-proline indicated this does not serve as a precursor for ornithine in *T. brucei* (I. Vincent and M.P. Barrett, unpublished data). It therefore seems that L-proline is not a precursor for polyamine biosynthesis in trypanosomatids.

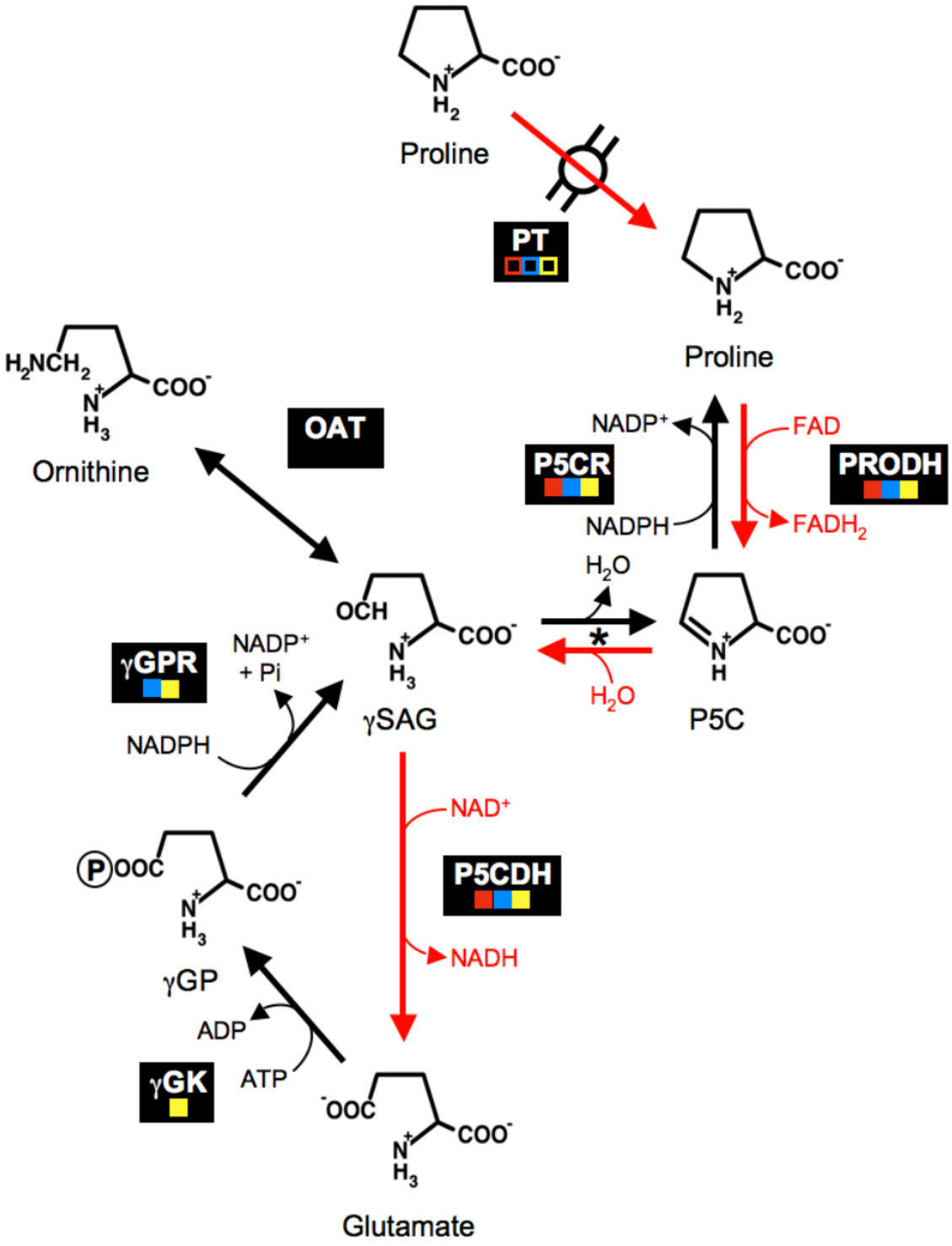

Figure 2. Reactions catalyzed by L-proline catabolic and biosynthetic enzyme (adapted from (102)). Red and black arrows correspond to enzymatic steps involved in the catabolic and biosynthetic pathways, respectively. Fully coloured squares indicate that the gene is present in *T. brucei* (red), *T. cruzi* (blue) and *L. major* (yellow) genomes. Empty coloured squares designate that L-proline transport (PT) activity was charaterized but the genes are unknown. The asterisk indicates a nonenzymatic reaction. For abbreviations and catabolic enzymatic steps, see Figure 3. Enzymes and abbreviations not shown in Figure 3: γGK, γ-glutamyl kinase; γGP, glutamate γ-semialdehyde; γGPR, γ-glutamyl phosphate reductase; OAT, ornithine aminotransferase; P5CR, pyrroline-5-carboxylate reductase; PT, L-proline transporter.

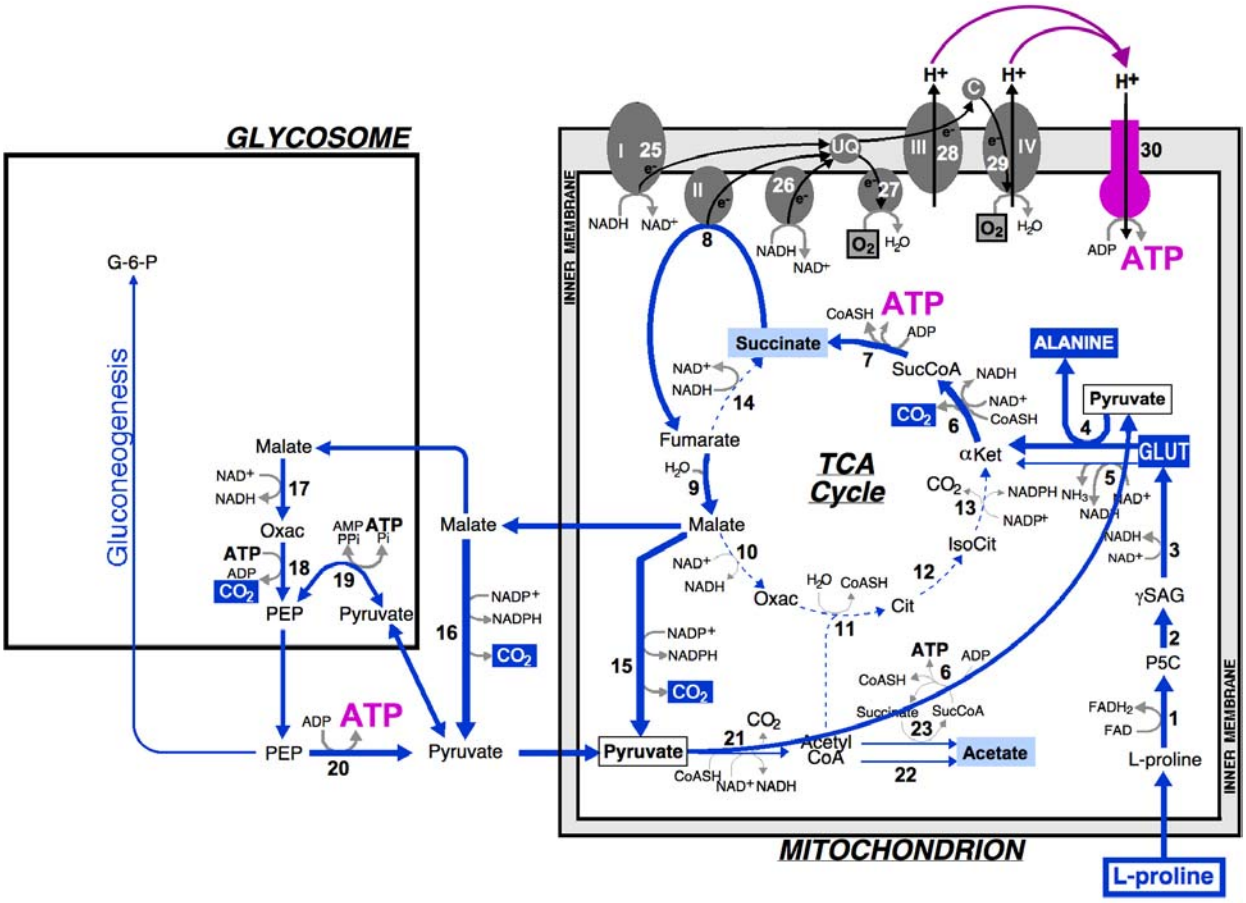

Figure 3. Schematic representation of L-proline metabolism in procyclic *T. brucei* growing in glucose-depleted medium. Blue arrows represent enzymatic steps of L-proline metabolism. Excreted end products are in white characters on a blue background (major end products: L-alanine, L-glutamate and CO_2) or in black characters on a light blue background (minor end products: acetate and succinate). At reversible steps, only the presumed or demonstrated direction of the reaction is represented. Dashed arrows indicate steps considered to occur at background level or not at all under glucose-depleted growth conditions. The glycosomal and mitochondrial compartments, the tricarboxylic acid cycle (TCA cycle) and gluconeogenesis are indicated. Abbreviations: C, cytochrome *c*; Cit, citrate; CoASH, coenzyme A; DHAP, dihydroxyacetone phosphate; G-6-P, glucose-6-phosphate; GLUT, glutamate; Gly-3-P, glycerol-3-phosphate; IsoCit, isocitrate; 2Ket, 2-ketoglutarate; Oxac, oxaloacetate; P5C, pyrroline-5-carboxylate; PEP, phosphoenolpyruvate; Pi, inorganic phosphate; PPi, inorganic pyrophosphate; γSAG, glutamate γ-semialdehyde; SucCoA, succinyl-CoA; UQ, ubiquinone pool. Enzymes: 1, proline dehydrogenase (PRODH); 2, spontaneous reaction; 3, pyrroline-5 carboxylate dehydrogenase (P5CDH); 4, L-alanine aminotransferase (AAT); 5, glutamate dehydrogenase (GDH); 6, α-ketoglutarate dehydrogenase complex; 7, succinyl-CoA synthetase (SCoAS); 8, succinate dehydrogenase (SDH; complex II of the respiratory chain); 9, mitochondrial fumarase; 10, mitochondrial malate dehydrogenase; 11, citrate synthase; 12, aconitase; 13, NADP-dependent isocitrate dehydrogenase; 14, mitochondrial NADH-dependent fumarate reductase (FRDm); 15, mitochondrial malic enzyme (MEm); 16, cytosolic malic enzyme MEc); 17, glycosomal malate dehydrogenase; 18, phosphoenolpyruvate carboxykinase (PEPCK); 19, pyruvate phosphate dikinase (PPDK); 20, pyruvate kinase (PYK); 21, pyruvate dehydrogenase complex; 22, unknown enzyme; 23, acetate:succinate CoA-transferase (ASCT); 25, complex I of the respiratory chain; 26, rotenone-insensitive NADH dehydrogenase; 27, alternative oxidase (AOX); 28, complex III of the respiratory chain; 29, complex IV of the respiratory chain; 30, F_0F_1-ATP synthase (ATPε).

After converting L-proline into L-glutamate, in trypanosomatids this product is catabolized further into the excreted end products succinate and/or L-alanine using enzymatic activities from central metabolic pathways, including enzymes that participate in the TCA cycle. Glucose availability in the growth medium determines which of these end products are generated. For instance, L-proline is converted primarily into succinate in glucose-rich conditions. In contrast, succinate is metabolized further into L-alanine by wild type procyclic trypanosomes growing in the absence of glucose (Figures 3 and 4) (27).

5.3. Proline degradation in the absence of glucose

5.3.1. Conversion of proline into alanine

Theoretically L-glutamate can be converted into the TCA cycle intermediate α-ketoglutarate by two

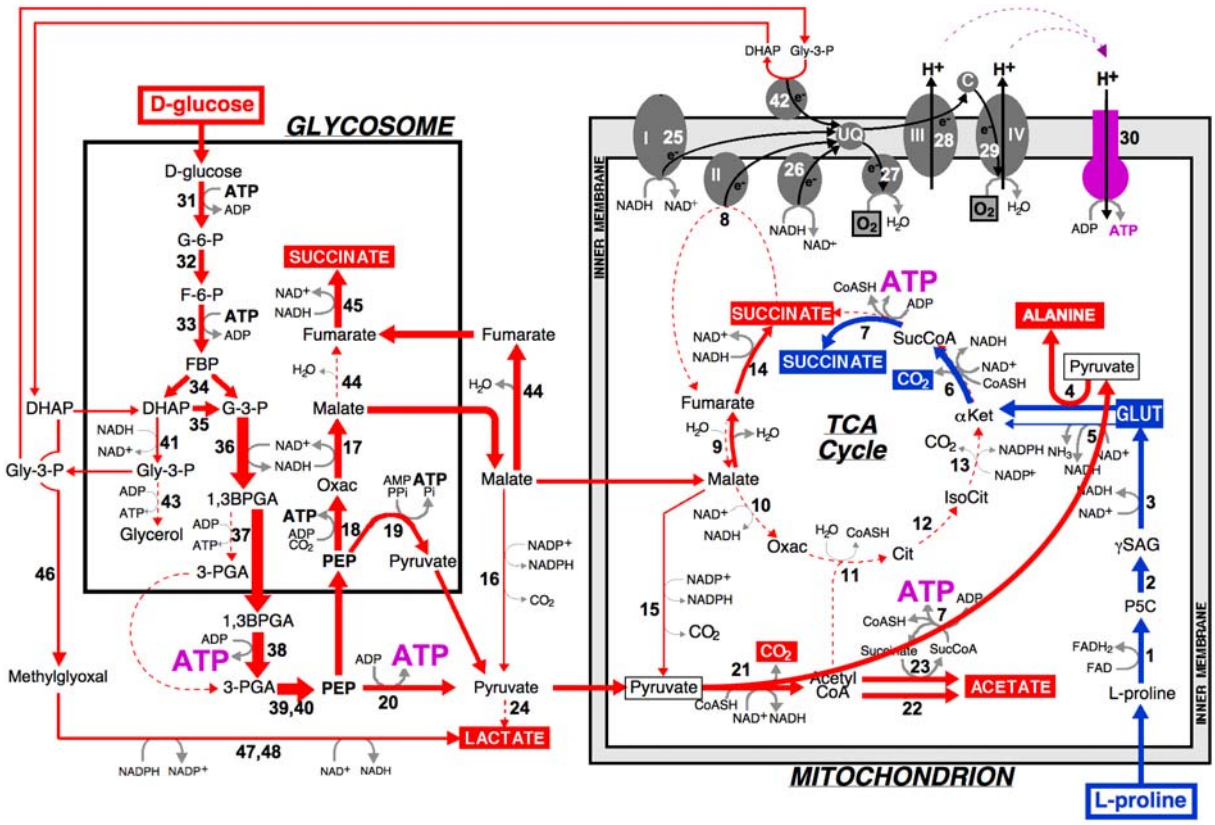

Figure 4. Schematic representation of L-proline and glucose metabolism in procyclic *T. brucei* growing in glucose-rich medium. See Figure 3 for the legend. Red arrows represent enzymatic steps of glucose metabolism. Excreted end products are in white characters on a red background. Abbreviations not used in Figure 3: 1,3BPGA, 1,3-bisphosphoglycerate; F-6-P, fructose-6-phosphate; FBP, fructose-1,6-bisphosphate; G-3-P, glyceraldehyde-3-phosphate; Gly-3-P, glycerol-3-phosphate; 3-PGA, 3-phosphoglycerate. Enzymes not shown in Figure 3: 31, hexokinase: 32, glucose-6-phosphate isomerase; 33, phosphofructokinase; 34, aldolase; 35, triose-phosphate isomerase; 36, glyceraldehyde-3-phosphate dehydrogenase; 37, glycosomal phosphoglycerate kinase; 38, cytosolic phosphoglycerate kinase; 39, phosphoglycerate mutase; 40, enolase; 41, NADH-dependent glycerol-3-phosphate dehydrogenase; 42, FAD-dependent glycerol-3-phosphate dehydrogenase; 43, glycerol kinase; 44, cytosolic fumarase; 45, glycosomal NADH-dependent fumarate reductase (FRDg); 46, nonenzymatic reaction; 47, NADPH-dependent methylglyoxal reductase; 48, NAD^+-dependent L-lactaldehyde dehydrogenase.

enzymatic activities, alanine aminotransferase (AAT, step 4) or mitochondrial L-glutamate dehydrogenase (GDH, step 5). Evans and Brown formulated a pathway for L-proline catabolism in procyclic trypanosomes whereby AAT catalyses the transamination of L-glutamate and pyruvate with concomitant production of α-ketoglutarate and L-alanine, the latter being excreted (107). The involvement of AAT was also proposed for L-alanine production from L-proline in the *T. congolense* procyclics (100). Most of the AAT activity in procyclic trypanosomes is encoded by the *AAT* gene, as demonstrated by the 95% reduction in AAT activity observed in an ^{RNAi}AAT mutant cell line (108). A cytoplasmic aspartate aminotransferase in *T. brucei* also possesses AAT activity (109), but its contribution to total cellular AAT activity is low. A recent study that combined gene knockout, RNAi and NMR spectroscopy confirmed that AAT activity is critical for generating L-alanine from L-proline (108) and essential for viability of the procyclic form of *T. brucei*, regardless of the presence of glucose. The finding that *AAT* gene deletion cannot be tolerated by procyclic trypanosome forms suggests that any contribution of GDH to L-proline degradation is negligible. Nevertheless, it has been speculated that GDH is involved in L-proline catabolism in *T. cruzi* (110). An alternative possibility is that the relatively high GDH activity observed in trypanosomatids is involved in a reverse reaction that synthesizes L-glutamate; a reaction reported to occur in some prokaryotes (111) that encode GDH orthologs closely related to the trypanosomatid GDH gene (112). Of note, the trypanosomatid genomes sequenced to date contain a single *GDH* gene, shown in *L. tarentolae* promastigotes to encode a mitochondrial protein (113). Importantly, given the characteristic excretion of L-glutamate from most insect trypanosomatid forms, whichever enzymatic activity (AAT with/without GDH) produces α-ketoglutarate from L-glutamate, that reaction is rate limiting and under metabolic control. In line with this premise, NMR analysis of

excreted end products from [^{13}C]-L-proline metabolism of procyclic trypanosomes incubated in PBS established that addition of a large excess of pyruvate (the possible limiting substrate in these experimental conditions) does not stimulate degradation of [^{13}C]-L-proline-derived L-glutamate (27).

α-Ketoglutarate is metabolized further in a series of enzymatic transitions that are part of the TCA cycle and is converted into malate (Figure 3, steps 6-9), which is in turn metabolized further in parallel reactions taking place in different metabolic compartments. This series of TCA enzymes is essential for trypanosomes grown in glucose-depleted conditions, as indicated by the low amount of malonate (a metabolic inhibitor of succinate dehydrogenase, SDH, step 8) required to kill procyclic trypanosomes and the lethal phenotype of the ^{RNAi}SDH procyclic mutant cell line (27). It is important to note that in procyclic trypanosomes (and probably in other trypanosomatids) the TCA cycle does not function as a cycle even though all the TCA cycle enzymes are expressed; most acetyl-CoA produced in the mitochondrion is converted into acetate (see below) (101, 114). Consequently, the flux through citrate synthase, the initial step of the TCA cycle (step 11), is relatively low compared to the metabolic flux due to L-proline breakdown. A corollary of this is that the α-ketoglutarate/malate section of the TCA cycle functions primarily to degrade L-proline, regardless of the amount of glucose in the medium.

Malate is converted into pyruvate (steps 15-20), which is the substrate required alongside L-glutamate for AAT to produce L-alanine and α-ketoglutarate earlier in the pathway (step 4). Three different pathways involving three subcellular compartments can produce pyruvate: the mitochondrial malic enzyme (MEm, step 15); the cytosolic malic enzyme (MEc, step 16); and a three-step pathway involving two glycosomal enzymes, malate dehydrogenase (step 17) and phosphoenolpyruvate carboxykinase (PEPCK, step 18), and the cytosolic pyruvate kinase (PYK, step 20). The final reaction of the latter pathway can also be performed by the glycosomal pyruvate phosphate dikinase (PPDK, step 19), however this PPi-dependent enzyme also catalyses the reverse reaction that produces PEP from pyruvate to feed gluconeogenesis (115), as observed in C4-plants and *Propionibacterium shermanii* (116). Notably, at least one of the two ME isoforms is essential for procyclic trypanosomes, since simultaneous RNAi silencing of both genes causes irreversible cell death regardless of glucose conditions (27). In glucose-depleted conditions the essential role of ME could be in L-alanine production, although it seems likely that the glycosomal pathway should compensate downregulation of the cytosolic and mitochondrial pathways in the ^{RNAi}MEc/m double mutant. Indeed, there is evidence that the glycosomal branch (steps 17-20) participates in pyruvate production in wild type cells, as the ^{RNAi}PEPCK (step 18) mutant exposed to [^{13}C]-L-proline excretes more [^{13}C]-L-aspartate, reflecting accumulation of oxaloacetate, the substrate of both PEPCK and the L-aspartate-producing enzyme (L-aspartate aminotransferase) (27). Therefore, it appears more likely that the essential role of ME in procyclic trypanosomes is in NADPH production, which is required to maintain cellular redox potential.

5.3.2. Minor end products of proline metabolism

NMR analyses of [^{13}C]-enriched metabolites excreted by procyclic trypanosomes exposed to [4-^{13}C]-L-proline identified two minor end products of L-proline metabolism, acetate and ß-hydroxybutyrate, each of which are associated with acetyl-CoA metabolism (27). Acetate, a major end product of glucose metabolism, is excreted by all insect trypanosomatid forms analysed so far (86, 101, 117-121), as well as most intracellular and extracellular mammalian forms (117, 120, 122) and the plant trypanosomatid *Phytomonas* spp. (123). As mentioned above, acetyl-CoA produced in the mitochondrion from pyruvate is not further metabolised in the TCA cycle (101), but converted into acetate by acetate:succinate CoA-transferase (ASCT, step 23), which transfers the CoA group of acetyl-CoA to succinate to produce acetate and succinyl-CoA (124, 125). The ASCT reaction is linked directly to ATP production through succinyl-CoA synthetase (SCoAS, step 7), which converts succinyl-CoA back into succinate. This ASCT/SCoAS cycle is also a feature of other parasites, such as trichomonads and helminths (126, 127). In procyclic trypanosomes, an additional unknown enzyme is involved in acetate production, since deletion of the *ASCT* gene does not totally abolish acetate production from glucose (125).

It is noteworthy that L-threonine is an essential carbon source for the EATRO1125 procyclic trypanosome strain (C. Ebikeme and F. Bringaud, unpublished data). L-Threonine appears to be converted in the mitochondrion into L-glycine and acetate (pathway not described in Figures 3 and 4) (128), the latter probably generated by the same enzymes that generate acetate from glucose (steps 22 and 23). Although amino acids other than L-proline do not sustain growth in glucose- and L-proline-depleted media, L-threonine is consumed in relatively high quantities by procyclic trypanosomes regardless of the extracellular concentration of glucose. Indeed, procyclic trypanosomes consume more L-threonine than L-proline even when glucose is absent (26). It was proposed that the key role of L-threonine is to provide 2-carbon units for lipid biosynthesis (129). In line with this premise, acetate produced from glucose (and probably L-threonine) metabolism is utilized for *de novo* fatty acid biosynthesis in procyclic trypanosomes (130) *via* a process termed the 'acetate shuttle'. Specifically, acetate produced in the mitochondrion from acetyl-CoA moves to the cytosol where the 'AMP-forming' acetyl-CoA synthetase converts it back to acetyl-CoA at the expense of ATP. This shuttling is essential for *de novo* biosynthesis of lipids as RNAi silencing of acetyl-CoA synthetase is lethal for parasites. To date, the acetate shuttle has only been evidenced in trypanosomes and replaces the ubiquitous citrate shuttle, which does not operate in trypanosomatids (130).

ß-hydroxybutyrate, the other minor end product of L-proline metabolism, is a ketone body. It serves as a carbon source in mammals and for energy storage in prokaryotes in the form of poly-ß-hydroxybutyrate, but its

role in the metabolism of trypanosomatids remains unclear. It can be produced from acetyl-CoA, via the intermediate hydroxy-methylglutaryl-CoA (HMG-CoA), by a series of enzymatic reactions involving acetyl-CoA acetyltransferase, HMG-CoA synthase, HMG-CoA lyase and ß-hydroxybutyrate dehydrogenase; genes encoding each of these enzymes are present in the *T. brucei* genome. ß-hydroxybutyrate is also an excreted end product of glucose metabolism when acetyl-CoA accumulates and was considered previously a by-product of acetyl-CoA metabolism (90, 125).

NMR analyses of *T. brucei* procyclics (27) and enzymatic assays in procyclic *T. congolense* (100) reveal that acetate represents only 3.2% and 2.2% of the excreted metabolites from L-proline metabolism, respectively. Thus, it appears that flux through the 'acetate branch' of L-proline metabolism is low. This low flux can perhaps be attributed to the fact that the acetate required for feed fatty acid biosynthesis is likely derived from metabolism of L-threonine, which is the main carbon source in glucose-depleted conditions (26). ß-hydroxybutyrate represents 3.1% of the excreted metabolites from L-proline metabolism and it remains unclear why it is produced in these amounts, as L-proline-derived acetyl-CoA seems to be equally converted into ß-hydroxybutyrate and acetate.

5.4. Proline metabolism in glucose-rich medium

Cross *et al.* observed that procyclic trypanosomes consume L-proline at a low rate and glucose at a considerably higher rate when both carbon sources are present in the medium (131). Recent determinations of L-proline consumption in glucose-rich (glucose at 6-10 mM) *versus* glucose-depleted conditions have confirmed this finding and demonstrated a substantially increased rate of L-proline consumption (from 2 to 6-fold depending on the trypanosome strains used and experimental conditions) when the extracellular glucose concentration is below 200 µM (26-28, 132). In addition, NMR analyses of the end products of L-proline metabolism revealed that in the presence of glucose, [^{13}C]-L-proline is converted into [^{13}C]-L-glutamate and [^{13}C]-succinate, with [^{13}C]-L-alanine barely detectable (27, 90). Taken together, these data establish that glucose regulates L-proline metabolism, influencing not only the rate of L-proline consumption but also which metabolic pathway is active; specifically, in rich media the conversion of L-proline-derived succinate into L-alanine is prevented. In line with this, the ^{RNAi}SDH mutant cell has no growth phenotype in glucose-rich conditions, further corroboration that succinate produced from L-proline does not need to be metabolized (27). L-alanine is still excreted by the procyclic cells (26), but the pyruvate required to convert L-glutamate into α-ketoglutarate and L-alanine (AAT, step 4) is derived from glucose degradation and not from metabolism of L-proline-derived succinate (Figure 4). Similarly, in rich medium the essential metabolites of intermediary metabolism, including fumarate (133) and malate (malic enzyme activity is essential for growth (27)), are not derived from L-proline catabolism but produced during glucose metabolism.

Generally, little attention has been paid to L-proline metabolism in bloodstream forms of *T. brucei*, probably because the large glycolytic flux (5 to 10-fold higher than in procyclic trypanosomes) is considered sufficient to produce all the ATP required for anabolic pathways. Therefore it was surprising when recent studies uncovered that two enzymes in the L-glutamine/L-proline catabolic pathway, SCoAS and AAT, are essential for the bloodstream parasite (108, 134). We have recently shown (I. Vincent and M.P. Barrett, unpublished data) that heavy atom labelled L-proline enters bloodstream forms but is not converted into glutamate whilst in procyclic forms the heavy atom is traced into glutamate. By contrast glutamine is converted to glutamate in bloodstream forms while external glutamate does not enter bloodstream forms in detectable levels.

5.5. Glucose metabolism

5.5.1. Production of succinate and acetate

Procyclic trypanosomes convert glucose by aerobic fermentation into partially oxidized end products, such as succinate, acetate and lactate (for reviews see: (23, 80, 91, 92)) (Figure 4). Most of glycolysis takes place in specialized peroxisomes, called glycosomes (steps 31-36) (24). In the course of glycolysis, phosphoenolpyruvate (PEP) is produced in the cytosol (steps 38-40), where it is located at a branching point to feed the glycosomal 'succinate branch' and the mitochondrial 'acetate and succinate' branches. To produce malate and fumarate from glucose, PEP must re-enter the glycosomes where it is converted into succinate within that compartment (steps 17, 18, 45) with the involvement of the cytosolic fumarase (step 44). Malate that moves from the glycosomes into the mitochondrion can also be converted into succinate therein (steps 9 and 14). Additionally, PEP can be converted by cytosolic PYK (step 20) and/or glycosomal PPDK (step 19) into pyruvate, which enters the mitochondrion and is converted to acetyl-CoA (step 21). As mentioned above the TCA cycle does not function as a true cycle (101) and acetyl-CoA is converted primarily into excreted acetate (steps 22 and 23) (124, 125). Finally, the ME reactions (steps 15 and 16) are essential even in the presence of glucose and constitute a bridge between the succinate and acetate branches (Figure 4) (27).

5.5.2. Maintenance of glycosomal balances

Within glycosomes, consumption and production of NADH is tightly balanced. NADH resulting from the reaction catalyzed by glyceraldehyde-3-phosphate dehydrogenase (step 36) must be re-oxidized inside the organelle. The glycosomal succinic fermentation pathway, which contains two NADH-dependent oxidoreductases, malate dehydrogenase (step 17) and fumarate reductase (FRDg, step 45) (121), is critical for maintaining redox balance (90). Theoretically, the glycosomal redox balance can be maintained also by the glycerol-3-phosphate (Gly3P)/dihydroxyacetone-phosphate (DHAP) shuttle (steps 41 and 42) (135). This shuttle comprises (*i*) a glycosomal NADH-dependent glycerol-3-phosphate dehydrogenase (step 41), which produces Gly3P from DHAP, (*ii*) a putative glycosomal exchanger, which exchanges Gly3P for DHAP between the glycosomal and cytosolic subcellular compartments, and (*iii*) the

mitochondrial FAD-dependent glycerol-3-phosphate dehydrogenase (step 42), which regenerates DHAP from Gly3P. Electrons produced by the mitochondrial FAD-dependent glycerol-3-phosphate dehydrogenase are ultimately transferred to molecular oxygen via the mitochondrial respiratory chain (steps 27-29). Insect forms express a functional Gly3P/DHAP shuttle but its contribution to maintaining the glycosomal redox balance is negligible in wild type parasites (90). It is however possible that the shuttle provides beneficial metabolic flexibility under certain growth conditions (135). In contrast bloodstream forms depend on the Gly3P/DHAP shuttle to balance the glycosomal NAD^+/NADH ratio, since these forms excrete pyruvate as the exclusive end product of glycolysis and do not express enzymes of the 'succinate branch' (5, 136).

Similarly, the glycosomal ADP/ATP balance must be tightly controlled in trypanosomatids. Several studies indicated that compartmentalization of glycolysis protects the parasite from 'run-away' metabolism due to the unregulated nature of the hexokinase (step 31) and phosphofructokinase (step 33) enzymes (87, 137-139). Compartmentalisation ensures these enzymes utilize only the ATP available within the glycosomes, and as a consequence, ATP consumed by these two enzymes must be regenerated inside the glycosomes by other kinases. Currently (Figure 4), PEPCK (step 18) is considered the main kinase involved in regenerating ATP. However, glycolysis continues to function in a Δ*pepck* procyclic cell line, albeit at a reduced rate, indicating that an alternative enzyme can regenerate ATP (90). Glycosomal phosphoglycerate kinase (Figure 4, step 37) serves to regenerate ATP in bloodstream *T. brucei* forms but not in procyclic forms, as in the latter this enzymatic activity is relocated to the cytosol (step 38) (140). Similarly, glycerol kinase (step 43) regenerates ATP in anaerobically grown bloodstream *T. brucei* forms, but its activity is marginal in the Δ*pepck* cell line (90). It is possible that the metabolic flux is redistributed to the glycosomal PPDK (step 19) and in this way ATP is regenerated in the absence of PEPCK. Future studies should delineate how the glycosomal ATP/ADP balance is maintained in the Δ*pepck* cell line to gain insight into the metabolic flexibility of insect form parasites.

5.5.3. Lactate production

Procyclic trypanosomes, like most trypanosomatids, produce lactate from glucose, although often as a minor end product (80, 92). The promastigote form of *L. braziliensis* (as well as the other *Leishmania* spp.) express the glyoxalase system, which serves as a detoxification pathway to protect the cell from damage by methylglyoxal (141), a toxic compound formed from DHAP mainly in the cytosol as a by-product of glycolysis. This system comprises glyoxalase I and glyoxalase II, which convert methylglyoxal into D-lactate using trypanothione as a cofactor (142). Notably, the *T. brucei* genome appears to encode only the glyoxalase II gene, suggesting that a canonical glyoxalase system is not functional in *T. brucei* (143). This premise was confirmed recently when it was demonstrated that expressing the *T. cruzi* glyoxylase I gene in the bloodstream form of *T. brucei* resulted in a functional glyoxylase system (144). Furthermore, in the same study it was shown that both *T. brucei* forms detoxify methylglyoxal using a pathway that produces L-lactate, instead of D-lactate, and involves NADPH-dependent methylglyoxal reductase and NAD^+-dependent L-lactaldehyde dehydrogenase activites (steps 47 and 48). These data are consistent with the observation that *T. brucei gambiense* procyclics only excrete the L-lactate isomer and do not produce D-lactate (141). This methylglyoxal detoxification pathway is probably the only source of L-lactate, since the *T. brucei* genome does not encode a lactate dehydrogenase gene and L-lactate dehydrogenase activity is undetectable in *T. brucei* procyclics.

Considering that methylglyoxal-derived lactate is a by-product of glycolysis, the amount of lactate produced should depend on glycolytic flux. In line with this, lactate excretion is considerably reduced in mutant procyclic trypanosomes that exhibit reduced glucose consumption (27, 90, 121, 133, 145, 146). Interestingly, the rate of glucose consumption is increased ~20% and ~50% in the ^{RNAi}ASCT and gene knockout Δ*asct* mutant cell lies, respectively, without affecting the rate of lactate excretion (125).

5.6. Comparison of energy metabolism in glucose-rich and -depleted conditions

5.6.1. ATP production by oxidative phosphorylation *versus* substrate level phosphorylation

Like in other eukaryotes, in trypanosomatids ATP can be produced in two different ways, in the mitochondrion by oxidative phosphorylation and in all subcellular compartments by substrate level phosphorylation. Oxidative phosphorylation produces ATP by utilizing the energy released when nutrients are oxidized. The full enzymatic machinery for oxidative metabolism is present in insect trypanosomatid forms. Specifically, the energy released when electrons flow through the electron transport chain (steps 8, 25-29, 42) is used to transport protons across the inner mitochondrial membrane (steps 28 and 29). This store of energy is tapped by allowing protons to flow back across the membrane through the F_0F_1-ATP synthase (ATPε, step 30) with a net production of ATP in the mitochondrial matrix. In contrast, substrate level phosphorylation involves chemical reactions that promote the direct transfer and donation of a phosphoryl group to ADP from a reactive phosphorylated intermediate.

Classically oxidative phosphorylation and more specifically, the F_0F_1-ATP synthase, was considered to be the principal source of ATP during glucose-based energy metabolism in procyclic trypanosomes (147). However, it has become clear that the role of F_0F_1-ATP synthase in energy production is dispensable. Exposing procyclic trypanosomes to oligomycin (the most specific inhibitor of the mitochondrial F_0F_1-ATP synthase), at concentrations 10-fold higher than required to inhibit the rotary enzyme, was found to only moderately alter the intracellular ATP

concentration or growth phenotype of parasites grown in glucose-rich conditions (27). In line with this, RNAi silencing of the expression of the subunit $F_1\beta$ of the F_0F_1-ATP synthase was observed to hardly affect the growth rate of the same procyclic strain (EATRO1125) (145). In contrast, in the same rich conditions, substrate level phosphorylation (steps 7, 18-20 and 38) was evidenced to be essential for ATP production. As mentioned above, PEPCK (step 18) and PPDK (step 19) participate in the maintenance of the glycosomal ADP/ATP balance and are not involved in net cellular ATP production (Figure 4). Therefore, the three enzymes responsible for net ATP production in procyclic parasites growing in glucose-rich conditions are: phosphoglycerate kinase (step 38), PYK (step 20) and SCoAS (step 7). Accordingly, Bochud-Allemann and Schneider demonstrated that the TCA cycle enzyme SCoAS is essential for ATP production and the viability of procyclic trypanosomes grown in glucose-rich conditions (148). This finding is not surprising given that SCoAS not only converts L-proline-derived succinyl-CoA into succinate during L-proline catabolism (step 7) but also regenerates succinate consumed by ASCT during glucose and L-threonine degradation (step 7, 23). The essential role of substrate level phosphorylation in glucose-rich conditions was confirmed when RNAi silencing of PYK, an enzyme involved only in glucose metabolism, was shown to be lethal for parasites growing in rich-medium but to hardly affect growth in the absence of glucose (88, 145). It is noteworthy that recent studies indicate that the mitochondrial F_0F_1-ATP synthase is essential for ATP production in glucose-rich conditions in some strains (149).

In contrast, ATP production by oxidative phosphorylation is essential for procyclic trypanosomes growing in glucose-depleted medium. Accordingly, the EATRO1125 strain is highly sensitive to oligomycin when grown in glucose-depleted medium (~1,000-fold more sensitive than in glucose-rich conditions) and even partial RNAi silencing of F_0F_1-ATP synthase expression results in death (^{RNAi}ATPε cell line) (27, 145).

5.6.2. Respiratory chain activity is essential

As mentioned ATP production by oxidative phosphorylation is essential for procyclic trypanosomes growing in glucose-depleted medium and thus respiratory chain activity is essential under these conditions. Respiratory chain activity is also required in the presence of glucose, although the F_0F_1-ATP synthase is not essential (at least in the EATRO1125 strain). Accordingly, inhibition of both terminal oxidases, the cyanide-sensitive enzyme (step 29) and the plant-like alternative oxidase (AOX, step 27), by a combination of cyanide and salicylhydroxamic acid (SHAM), respectively, has a dramatic effect on cell viability (101, 145). Furthermore, all insect trypanosomatid forms grown in rich conditions arrest growth and eventually die in the absence of oxygen (101, 150, 151). Respiratory chain activity is essential in trypanosomatids irrespective of glucose conditions, as in most eukaryotes, because it is necessary for purposes in addition to oxidative phosphorylation, such as oxidation of the reducing equivalents (NADH and $FADH_2$/$FMNH_2$) produced in intermediary metabolism. Theoretically, in most trypanosomatids electrons can be provided to the respiratory chain by three FAD-dependent enzymes (steps 1, 8 and 42) and two NADH dehydrogenases (steps 25 and 26) involved in glucose and L-proline metabolism (Figure 4). Two of the FAD-dependent enzymes, PRODH (step 1) and SDH (step 8), are involved in L-proline catabolism, the latter required only in the absence of glucose, and the third, mitochondrial glycerol-3-phosphate dehydrogenase (step 42), participates in glucose metabolism, but only in a limited way in procyclic trypanosomes (90).

5.6.3. Mitochondrial NADH dehydrogenase activities

In procyclic trypanosomes both NADH dehydrogenases are associated with the mitochondrial inner membrane. The rotenone-insensitive NADH dehydrogenase (step 26) oxidizes NADH without proton translocation across the membrane (152). The function of rotenone-sensitive NADH, called ubiquinone oxidoreductase (complex I of the respiratory chain, step 25), and its contribution to energy metabolism, is the subject of debate (94, 153). For instance, the complex I activity observed in mitochondrial fractions exhibits an unusually low sensitivity to rotenone, and thus it has been speculated that the relatively high quantity of rotenone required to kill the parasite relates to its binding non-specifically to other electron carriers (154). Genes encoding 18 putative subunits of the typical mitochondrial complex I, which can be composed of up to 46 subunits in some species (155), are encoded in the genomes of trypanosomatids sequenced to date (156). Fifteen of these putative complex I proteins were evidenced in the mitochondrion of procyclic trypanosomes using mass spectrometry (157). These 18 genes encode all complex I subunits known to be involved in electron transport, however four membrane subunits involved in proton extrusion do not appear to be encoded in trypanosomatid genomes. In light of this observation Opperdoes and Michels proposed that complex I is not involved in energy transduction but in regeneration of mitochondrial NAD^+ (156). In accord with this hypothesis, RNAi silencing of three different complex I subunits affects NADH oxidation activity but not the mitochondrial membrane potential (158). Recently, an atypical mitochondrial complex containing only 6 of the 18 putative subunits of complex I was isolated from procyclic trypanosomes (159). Notably, this atypical complex I was associated with additional proteins involved in fatty acids biosynthesis (a fatty acyl carrier and two *trans*-2-enoyl-CoA reductases) as well as two proteins containing superoxide dismutase domains, suggesting that this NADH-ubiquinone oxidoreductase-like complex is multifunctional.

Turrens and colleagues proposed that NADH is reoxidized in the mitochondrion by an additional pathway; a cycle involving mitochondrial SDH and FRD activities (steps 8 and 14) (153, 160). In this hypothetical cycle, fumarate accepts electrons from NADH when being converted into succinate by FRDm and then succinate is oxidized back to fumarate by complex II of the respiratory chain (SDH). Subsequently, the electrons are transferred via the quinone pool and the respiratory chain to molecular oxygen. Further analyses are required to understand more

fully how NADH is oxidized in the mitochondrion of procyclic trypanosomes by the different mitochondrial NADH dehydrogenase activities.

The finding that complex I subunits involved in proton extrusion are not encoded by trypanosomatid genomes implies that complexes III and IV (steps 28 and 29) are solely responsible for generating the mitochondrial proton gradient. Nevertheless, RNAi downregulation of the expression of two different complex III subunits involved in electron transfer (ApoC1 and Rieske) was not found to affect growth or the mitochondrial membrane potential of procyclic *T. brucei* (161).

5.6.4. Electron flow within the respiratory chain depends on glucose availability

Regardless of which mitochondrial dehydrogenases feed the respiratory chain and which complexes are involved in proton extrusion, the generation of mitochondrial membrane potential depends on electron flow through the respiratory chain and consequently on the production of reducing equivalents such as NADH (steps 3, 5, 6, 10 and 21) and $FADH_2$ (steps 1, 8 and 42). In glucose-depleted conditions, the relatively high metabolic flux through the L-proline degradation pathway produces significant quantities of reducing equivalents in the mitochondrion (steps 1, 3, 6 and 8), which are reoxidized mainly by the respiratory chain. Consequently, under these conditions the membrane potential generated by the respiratory chain appears sufficient to maintain essential mitochondrial functions and to produce by oxidative phosphorylation most of the ATP required.

The situation is different in procyclic trypanosomes growing in glucose-rich conditions. Firstly, given the 6-fold decrease in L-proline consumption and its conversion into succinate (instead of L-alanine), we estimate that about ten times fewer reducing equivalents (NADH and $FADH_2$) are produced in the mitochondrion. Secondly, the aerobic fermentation strategy employed by insect trypanosomatid forms means that glucose metabolism does not produce significant amounts of reducing equivalents, despite the relatively high glycolytic flux. According to the current understanding of procyclic metabolism (Figure 4), when glucose is being converted to succinate and acetate most of the reducing equivalents produced in the mitochondrion must be generated by the pyruvate dehydrogenase complex (step 21), since, (*i*) the involvement of the Gly3P/DHAP shuttle (steps 41-42) is negligible (90) and (*ii*) contribution of the flux through the mitochondrial malate dehydrogenase (step 10) is probably low (146). Notably, the flux through the acetate-producing branch that produces NADH (pyruvate dehydrogenase complex, step 21) is estimated to be equivalent to the flux through the mitochondrial 'succinic fermentation' pathway, which is responsible for NADH consumption (NADH-dependent fumarate reductase, FRDm, step 14) (146). Therefore, theoretically in glucose-rich conditions procyclic trypanosomes can maintain the mitochondrial redox balance without any significant contribution from the NADH-dehydrogenase activities of the respiratory chain. Thus, in rich medium, aerobic fermentation of glucose combined with the lack of a Pasteur effect (inhibition of glucose metabolism by oxygen), similar to the Warburg effect in tumor cells (glucose fermentation in the presence of oxygen), and downregulation of L-proline metabolism, leads to a considerable reduction of electron flow through the respiratory chain. This observation is consistent with the aforementioned reduced involvement of oxidative phosphorylation in ATP production when procyclics are growing in glucose-rich conditions.

Of note, trypanosomes express a mitochondrial plant-like alternative oxidase (AOX, step 27), which is not coupled to proton translocation and so is not involved in ATP production by oxidative phosphorylation (162). The availability of two terminal oxidases, one protonogenic and the other not, provides powerful flexibility to manipulate the electron flux through different respiratory chain branches in response to the redox state of the quinone pool. Thus, it is possible that when the demand for ATP production by oxidative phosphorylation is low, such as in glucose-rich conditions, the electron flow is redistributed toward AOX, preventing electron leakage from the mitochondrial chain (responsible for toxic production of reactive oxygen species). In support of this hypothesis, RNAi silencing of AOX leads to increased superoxide production and protein oxidation (163). An extension of this hypothesis is that in response to glucose depletion, electron flow is redistributed away from AOX toward the protonogenic complexes III and IV of the respiratory chain to enable increased oxidative phosphorylation activity. Summarily, like the equivalent plant enzyme, trypanosomal AOX likely participates in balancing the redox state of the quinone pool and facilitates flexible control of ATP synthesis to sustain growth rate homeostasis (164).

5.7. Regulation of the metabolic switch

Two key questions are, which steps of the L-proline pathway are responsive to glucose availability and which signal or signalling pathway(s) is used to regulate L-proline degradation and oxidative metabolism in procyclic trypanosomes. L-proline uptake and PRODH activity are ~3- and ~2-fold upregulated upon glucose depletion, respectively (26). Therefore, it can be surmised that at least the two first steps of the L-proline metabolic pathway are controlled by glucose, although other enzymatic steps of intermediary metabolism are probably also affected.

An obvious candidate for signalling a metabolic switch is glucose itself. This hypothesis is supported by the graded, rather than binary, nature of metabolic adaptation observed in response to glucose availability (132). More specifically, L-proline consumption exhibits a dose-dependent response to extracellular D-glucose concentration, indicating a gradual replacement of glucose metabolism by L-proline metabolism. Similarly, oligomycin sensitivity decreases gradually as a function of glucose concentration. Assuming D-glucose triggers the metabolic switch, the next question is what senses and transduces extracellular glucose levels. It is known that nutrient transporters and G protein-coupled receptors can function as extracellular sensors for carbon sources. For example, the yeast genome encodes 20 hexose transporters

(HXT), including the glucose sensors SNF3 and RGT2, which control expression of the other HXT family members (165). *T. brucei* expresses two characterized glucose transporters, the low affinity THT1 and the high affinity THT2, which are responsible for D-glucose uptake in the bloodstream and procyclic forms, respectively (166-168). Since THT2 has high affinity it is saturated by the time extracellular glucose reaches a few hundred micromolar, a concentration at which the gradual metabolic shift is still occurring, hence THT2 is not likely to be the glucose sensor in procyclics. However, it remains possible that the low affinity THT1, though not responsible for glucose uptake, serves as the glucose sensor in procyclic trypanosomes. Alternatively, or in addition, the sensor could be another protein related to the THT glucose transporters (called THT3), encoded by a gene discovered recently during completion of the *T. brucei* genome project.

Extracellular glucose itself cannot be the only signal. When in large excess the non-metabolisable glucose analogue N-acetyl-glucosamine (169), which inhibits D-glucose uptake without itself being internalized (170), induces parasites to shift metabolically toward oxidative degradation of L-proline, likely due to the sensing of diminished glucose uptake and/or reduced glycolytic flux (132). Likewise, various mutant cell lines with impaired glucose metabolism exhibit a shift toward oxidative degradation of L-proline irrespective of extracellular glucose levels. For example, the Δ*pepck* mutant that has compromised 'succinate branches' displays a 3-fold reduction in glycolytic flux and an associated 2-fold increase in the rate of L-proline consumption, with half of the L-proline-derived succinate converted into L-alanine (90). Similarly, the single ^{RNAi}FRDg and double ^{RNAi}FRDg/m mutants are characterized by a ~2-fold reduction in the rate of glucose consumption and an associated increase in L-proline metabolism (146).

In light of such data, we conclude that both glucose availability and glucose metabolism regulate metabolic switching by trypanosomatids. With regards to glucose metabolism, intermediary metabolites or end products of glucose metabolism are candidates for switch signals.

6. PROLINE RACEMASES

Many parasites, including the trypanosomatids, induce a degree of immunosuppression in their mammalian hosts. A possible mechanism underlying this phenomenon is polyclonal activation of B cells, which has long been known to occur during *T. cruzi* infection (171) and is suspected to contribute to auto-reactive responses during Chagas disease (172). In an effort to identify parasite proteins that stimulate lymphocyte growth, Reina-San-Martin and colleagues fractionated medium from *T. cruzi* cultures using HPLC and anion exchange chromatography and tested fractions in a lymphocyte proliferation assay (173). A 45 kDa protein was identified as stimulating B cell proliferation. Peptide sequencing and subsequent isolation of the corresponding full-length gene revealed that this protein has significant homology to bacterial proline racemases. Heterologous expression of the gene in *E. coli* and purification of the recombinant protein enabled confirmation that the protein does indeed possess proline racemase activity and is capable of interconverting D- and L-proline. This was the first proline racemase to be identified in a eukaryote. The protein interconverts only free proline and is not able to modify amino acids incorporated into peptide chains (174). Specific inhibitors, such as pyrrole-2 carboxylate, inhibit the racemase activity (173). In accordance with the rationale of its isolation, the recombinant protein is capable of stimulating B cell growth and this mitogenic activity can be inhibited physically (*e.g.* by heating) and chemically (173), which led to a suggestion it may be the enzymatic activity that stimulated B cell mitogenesis. However, site directed mutagenesis of the active site L-cysteine residues was found to inactivate racemase activity without abrogating mitogenic activity (175). In light of these data, it is speculated that transiently exposed epitopes, which are exposed in native, ligand-unbound enzyme but not in denatured or ligand-bound enzyme, are responsible for stimulating mitogenesis.

It transpires that *T. cruzi* has two proline racemase genes, one encoding a cytosolic protein (TcPRACB) and the other a secreted protein (TcPRACA), although differential trans-splicing can remove the signal peptide and then TcPRACA is cytosolic (176). Secreted TcPRACA appears to be produced only by trypomastigote and metacyclic forms. Crystallization of TcPRACA at 2.1Å resolution revealed that the enzyme exists as a dimer with two active sites and exhibits overall similarity to the *E. coli* enzyme diaminopimelate epimerase (DapF) (175). Notably, each isoform displays high K_m values for proline, 29 mM and 75 mM for the A and B isoforms, respectively, indicating that the enzymes are relatively inefficient but unlikely ever to reach saturation. It cannot be excluded that these enzymes have other substrates, although hydroxyproline and other amino acids have been excluded as candidates (173).

In addition to the putative role in B cell mitogenesis, a number of other possible roles for these enzymes have been proposed. Antibodies against proline racemases diminish the ability of *T. cruzi* trypomastigotes to invade mammalian Vero cells in a dose-dependent fashion, implicating a role in cellular invasion (174). In line with this, the proline racemase inhibitor pyrrole-2 carboxylate inhibits invasion when applied to the parasites in a pre-incubation but not if applied to Vero cells alone before introduction of parasites. In the same study it was demonstrated that pyrrole-2 carboxylate also interferes with the rate at which intracellular amastigotes differentiate into trypomastigotes, pointing to another possible role for racemases in differentiation. However, it should be taken into consideration that pyrrole-2-carboxylate could be acting on targets other than the racemases. Further support for both the differentiation and invasion roles is provided by an earlier study (177) in which overexpression of TcPRAC was shown to promote differentiation from intracellular amastigotes into released trypomastigotes and also invasion of mammalian cells. Recently, definitive support for any of these roles was sought using an antisense

RNA strategy to silence *T. cruzi* racemases, but the manipulated parasites died and so it is not possible to infer anything about function from these experiments (177). A possible interpretation of the antisense RNA experiments is that the racemase genes are essential, but antisense data should be analyzed with caution.

Another proposed role for the proline racemases is to provide D-proline for incorporation into parasite proteins (178). Many bacteria incorporate D-alanine or D-glutamate into constituents of their cell walls and proteins containing D-amino acids are less susceptible to protease cleavage (179). Thus, it is possible that the racemases enable parasites to produce proteins that are resistant to host proteases. In line with this model, *T. cruzi* has been shown to possess significant quantities of protein containing D-proline (178). Future studies should address the hypothesis that D-proline-containing parasite proteins provide resistance to host proteases.

The availability of genome sequences for *T. cruzi*, *T. brucei*, several *Leishmania* spp. and other African trypanosomes including *Trypanosoma vivax* and *Trypanosoma congolense* (in progress) has enabled an analysis of the evolutionary conservation of proline racemases across the kinetoplastidae. Remarkably, other than *T. cruzi*, only *T. vivax* possesses a proline racemase gene. In common with *T. cruzi, T. vivax* induces polyclonal B cell activation, but given that other African trypanosomes without proline racemases also stimulate polyclonal B cell stimulation it is not possible to draw any conclusions about this putative role. More work is needed to understand the function of proline racemases in *T. cruzi* and *T. vivax*.

7. ROLES FOR PROLINE IN TRYPANOSOMATID DIFFERENTIATION

As mentioned above, there is evidence to suggest that *T. cruzi* proline racemases influence differentiation (174, 177). In addition, increasing L-proline levels (from 20 μM to 200 μM) in the medium supporting amastigote-infected mammalian CHO-K1 cells was shown to induce a 2 to 3-fold increase in the numbers of released trypomastigotes (180); it is not known if this effect involves the racemases. Furthermore, addition of 10 mM L-thiazolidine-4-carboxylic acid, a L-proline analogue, to cultured mammalian cells infected with *T. cruzi* amastigotes diminishes trypanomastigote production. Finally, L-proline (and also L-glutamine) has been proposed to regulate the life cycle of the veterinary African trypanosome *T. congolense*, specifically when epimastigotes differentiate into metacyclics (181). Taken together, these data point to an important signaling role for L-proline during trypanosome differentiation. More detailed and systematic future studies should investigate this possibility.

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Abbreviations: RNAi: RNA interference; HAT: Human African trypanosomiasis; TCA: tricarboxylic acid; DFMO: difluormethylornithine; RVD: regulatory volume decrease; PI3, phosphatidylinositol 3; P5C: Δ-pyrroline-5-carboxylate; PRODH: proline dehydrogenase; γSAG): glutamic semialdehyde; P5CDH: NAD-dependent P5C dehydrogenase; PutA: L-proline utilization A; OAT: ornithine aminotransferase; AAT: alanine aminotransferase; GDH: L-glutamate dehydrogenase; SDH: succinate dehydrogenase; MEm: mitochondrial malic enzyme; MEc: step 15); cytosolic malic enzyme; PEPCK: phosphoenolpyruvate carboxykinase; PYK: pyruvate kinase; PPDK: pyruvate phosphate dikinase; ASCT: acetate:succinate CoA-transferase; SCoAS: succinyl-CoA synthetase; HMG-CoA: hydroxy-methylglutaryl-CoA; PEP: phosphoenolpyruvate; FRDg: glycosomal fumarate reductase; Gly3P: glycerol-3-phosphate; DHAP: dihydroxyacetone-phosphate; ATPε: F_0F_1-ATP synthase; AOX: plant-like alternative oxidase; SHAM: salicylhydroxamic acid; HXT: hexose transporter; PRAC: Proline racemase.

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