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Membrane-Related Processes and Overall Energy Metabolism in *Trypanosoma brucei* and Other Kinetoplastid Species

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An electrochemical proton gradient exists across the plasma membrane and the mitochondrial membrane of the bloodstream form of *Trypanosoma brucei*. The membrane potential across the plasma membrane and the regulation of the internal pH depend on the temperature. *Leishmania donovani* regulates its internal pH and maintains a constant electrochemical proton gradient across its plasma membrane under all conditions examined. The mitochondrion of the *T. brucei* bloodstream form is energized, even though the reactions taking place in it do not result in net ATP synthesis and the Kreb's cycle and the respiratory chain are absent. Glucose is transported across the plasma membrane of *T. brucei* by a facilitated diffusion carrier, that can transport a wider range of substrates than its mammalian counterparts. Pyruvate exits the cell via a facilitated diffusion transporter as well. Conflicting evidence exists for the mechanism of glucose transport in *L. donovani*; biochemical evidence suggests proton/glucose symport, while facilitated diffusion is indicated by physiological data.

KEY WORDS: Kinetoplastids; membrane potential; glucose uptake; energy metabolism.

INTRODUCTION

Membrane-related processes play an essential role in the energy metabolism of any organism or cell. Even though the parasitic protists belonging to the kinetoplastids have a metabolism featuring many unusual properties, they are no exception to this rule. This review will discuss two membrane-related processes that have an important role in the overall energy metabolism: (i) the electrochemical proton gradient, and (ii) substrate, in particular glucose transport. The focus will be primarily on the bloodstream form of *Trypanosoma brucei*, but other trypanosomatid species will also be considered.

THE ELECTROCHEMICAL PROTON GRADIENT

The Plasma Membrane

Two membranes of *T. brucei* long slender (LS) bloodstream forms are reported to have an electrochemical proton gradient (Δp) across them: the plasma membrane and the mitochondrial membrane. The first study on this subject demonstrated a membrane potential ($\Delta\Psi$) of -100 to -160 mV across the plasma membrane, bu measuring the distribution of $^{137}Cs^+$ in the presence of valinomycin (Midgley, 1983). Inhibition of respiration-reduced accumulation of Cs^+, while the ionophore CCCP abolished it completely. Using tetraphenylphosphonium as a probe, the $\Delta\Psi$ was shown to depend both on the external pH and on the composition of the external medium, with values varying from about -50 mV at pH 6.0 in a Na^+ buffer to more than -150 mV at pH 8.0 in a K^+ buffer (Thissen and Wang, 1991). The internal pH was regulated at $pH_{in} = 7.2$ in the range of $pH_{out} = 6.0$ to 8.5. In a study utilizing two probes that should both measure membrane potential ($\Delta\Psi$) Rb^+ and methyltriphenylphosphonium ($MePh_3P^+$), widely different accumulation ratios were obtained: around 25 for Rb^+ and approximately 500 for $MePh_3P^+$ (Nolan and Voorheis, 1991). The accumulation of $MePh_3P^+$, but not of

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Rb^+, was affected by valinomycin. In the presence of valinomycin and K^+ the accumulation of both probes was strongly reduced. The effect of FCCP was similar to that of valinomycin, not reducing Rb^+ accumulation, but lowering the ratio of the $MePh_3P^+$ in/out by a factor of about 10. Upon permeabilization of the plasma membrane by "swell-dialysis" Rb^+ is no longer accumulated, but a FCCP-sensitive accumulation of $MePh_3P^+$ remained. The main conclusion of this study, that there is a Δp over the plasma membrane and over the membrane of an internal compartment, is not contradicted by other reports. However, the accumulation of Rb^+ in the absence of valinomycin remains puzzling, as membranes are generally not permeable to this probe in the absence of valinomycin, unless they contain a potassium channel (Nicholls and Ferguson, 1992), for which there is no evidence. An unusual aspect of the membrane physiology of *T. brucei* is the temperature dependence of Δp (Ter Kuile *et al.*, 1992). At temperatures below 28°C, $\Delta\Psi$ is around -150 mV and the internal pH is kept constant at $pH_{in} = 7$, while at 37°C, pH_{in} is not regulated and $\Delta\Psi$ is reduced to -40 mV. This change in membrane properties could be correlated to a discontinuity in the increase of the membrane fluidity occurring around 26°C. The reduction of the membrane potential cannot be explained simply by physical properties of the membrane, because increased fluidity usually reduces the leakiness of a membrane. It is therefore more likely to represent adaptation of the LS forms to the conditions encountered in the mammalian bloodstream form.

The Mitochondrion

The reactions taking place in the mitochondrion of the *T. brucei* long slender bloodstream form do not result in net ATP synthesis even though oxygen is consumed at high rates (Opperdoes, 1987). The insect form has functional mitochondria. A major difference between the mitochondria of the bloodstream form and the insect form is that cytochromes are absent in the former, but cytochrome *c* is present in the mitochondria of the procyclic form (Torri and Hajduk, 1988; Hajduk, this issue). The mitochondria of LS bloodstream forms were reported not to fluoresce when exposed to rhodamine 123 (10 μg/ml), a fluorescent probe used to estimate membrane potential, while procyclic forms do (Bienen *et al.*, 1991). Using an elegant photographic technique, Divo and coworkers showed that the mitochondrion of the LS form in fact does sequester rhodamine 123, but optical staining was obtained at tenfold lower concentrations (1 μg/ml) (Divo *et al.*, 1993). The ability to sequester all dye only when exposed to a lower concentration indicates that the membrane potential is less in LS forms than in insect forms, even though the opposite conclusion was drawn by Divo and coworkers. In several studies using metabolic inhibitors and ionophores it was shown that the mitochondrion of the LS forms builds up an electrochemical proton gradient, probably by the F_1F_0 ATPase operating in the direction of ATP hydrolysis (Nolan and Voorheis, 1992; Vercesi *et al.*, 1991; Divo *et al.*, 1993). In the insect form the flow of electrons through a respiratory chain creates a ΔP across the mitochondrial membrane, which is then used for ATP synthesis (Bienen *et al.*, 1991; Divo *et al.*, 1993). This conclusion is confirmed by lack of influence of salicylhydroxamic acid on the uptake of Rb^+ and $MePh_3P^+$ by isolated mitochondria, while oligomycin, an inhibitor of the F_1F_0-ATPase, did reduce $MePh_3P^+$ accumulation (Nolan and Voorheis, 1992). The accumulation of Rb^+ by isolated mitochondria in the absence of valinomycin is difficult to understand, particularly because in their earlier study (Nolan and Voorheis, 1991) it was concluded that the second compartment to accumulate $MePh_2P^+$, now identified as the mitochondrion, did not accumulate Rb^+.

The major difference between the mitochondrion of the LS form of *T. brucei* and that of the procyclic form is the absence of the Kreb's cycle and the respiratory chain in the first. The synthesis of DNA, RNA, and protein proceeds at normal rates (Hajduk, this issue; Benne, this issue). Thus, despite the absence of metabolic pathway such as the citric acid cycle and the respiratory chain, a Δp would be an absolute prerequisite for the import of cytosolically synthesized proteins and for maintaining the structural and functional integrity of the mitochondrion. In this manner the mitochondrion of the LS form with its limited role in metabolism also remains in a condition that allows it to be rapidly transformed into a fully functional form. In bloodstream form *T. brucei* the mitochondrion has no role in the maintenance of calcium homeostasis (Ruben *et al.*, 1991), while in *T. cruzi* the dissipation of the mitochondrial membrane potential resulted in the release of calcium from the mitochondrion (Docampo *et al.*, 1993). Otherwise, the membrane potential of the *T. cruzi* mitochondrion was in the same range as that

reported for *T. brucei*, approximately −135 mV (Vercesi *et al.*, 1991).

A comparison of the membrane physiology of *T. brucei* and *Leishmania* species is particularly interesting. *Leishmania* spp reside in environments that are far more demanding than the mammalian bloodstream. In the macrophage and in the midgut of the sandfly, *L. donovani* is exposed to widely different pH, pH 4.5–5.0 and pH 7.0–7.5, respectively. It adapts to these environmental changes by maintaining a constant internal pH in a narrow range around pH 6.5 (Zilberstein *et al.*, 1989) or pH 7.0 (Glaser *et al.*, 1988). In the Ph range of 5.0 not only the internal pH remains constant, but the electrochemical proton gradient across the plasma membrane as well (Zilberstein *et al.*, 1989). The reported value of −90 to 100 mV is slightly lower than the values reported for LS *T. brucei* below 30°C. At pH 7.0 the membrane potential is between −90 and −113 mV (Glaser *et al.*, 1992), confirming the value for Δp, because there is no Δ pH at pH 7.0. Direct measurements of the Δp across the mitochondrial membrane have not been reported, but since the mitochondria of *Leishmania* spp play a much larger role in the energy metabolism than that of LS *T. brucei* (Blum, 1993), it is likely that their membranes are energized.

Little is known about the properties of the glycosomal membrane. The glycosome synthesizes but also consumes ATP, and is not involved in net ATP generation. No information is available indicating whether or not it is energized. To which substrates and intermediates it is permeable is not documented, and this must be considered as a severe gap in our understanding of the metabolism of the trypanosomatidae.

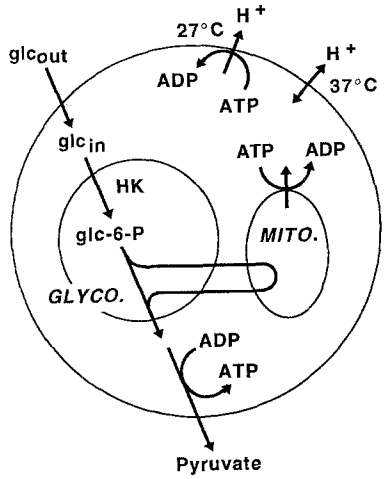

Fig. 1. *Trypanosoma brucei* bloodstream forms. Scheme indicating the major steps of glucose metabolism involving membranes and the distribution of the electrochemical proton gradient across membranes at 37°C and 27°C. The mechanisms for maintaining the proton gradient across the plasma membrane are not known.

GLUCOSE TRANSPORT

It has been established by many authors independently that glucose is taken up by the bloodstream form of *T. brucei* by a mechanism of facilitated diffusion (Gruenberg *et al.*, 1978; Eisenthal *et al.*, 1989; Ter Kuile and Opperdoes, 1991; Seyfang and Duszenko, 1991). In agreement with this, a gene coding for a protein that shows sequence homology with the human erythrocyte glucose transporter, which is a facilitated diffusion carrier, has been cloned from *T. brucei* (Bringaud and Baltz, 1992). The *T. brucei* carrier transports a wider range of substrates than the mammalian counterpart. Glucose, fructose, and mannose were readily transported, while L-sorbose inhibited fructose and glucose transport, but with a K_i exceeding the K_m of the other substrates by a factor of 10 (Eisenthal *et al.*, 1989; Fry *et al.*, 1993). A large variety of glucose analogues is acceptable, but substitutions at the C-3 and C-4 positions generally cause a greatly increased K_m, suggesting that the transporter creates hydrogen bonds at the C-3 and C-4 positions (Eisenthal *et al.*, 1989). D-Glucose and its analogues are transported in the pyranose form, but D-fructose is accommodated as a furanose ring (Fry *et al.*, 1993). In the model presented by Fry and coworkers, hydrogen-bonding side chains of the transporter are directed at the C-3, C-4, and C-5 and ring oxygen positions. This scheme allows for major substitutions at the C-2 position, as are indeed found experimentally.

The high metabolic rates of LS *T. brucei* are in part achieved by using facilitated diffusion as uptake mechanism for glucose and also as exit mechanisms for the end product pyruvate (Wiemer *et al.*, 1992; Barnard *et al.*, 1993). Facilitated diffusion carriers in general have more rapid turnover rates than active carriers (Stein, 1986), and, just as important for an organism that synthesizes only 2 ATP per glucose, do not require input of energy. From a physiological point of view, it would have been logical if the efflux of pyruvate would have been coupled to the expulsion of protons, thus creating an electrochemical proton gradient without metabolic costs. In the bloodstream this apparently does not occur, since no meaningful

Δp was measured (Ter Kuile *et al.*, 1992). Two explanations are possible: (1) In order to build up a sufficiently large pyruvate concentration gradient the internal concentration in the cell would have to be harmfully high. (2) The slower efflux through a coupled carrier might slow down the metabolic rate.

There are considerable variations in the kinetics reported for the glucose transporter of LS *T. brucei*. The values reported for the K_m for D-glucose start at 0.39 mM (Eisenthal *et al.*, 1989), and the highest value reported is 1.98 mM (Ter Kuile and Opperdoes, 1991). The V_{max} found experimentally depends on the conditions during the experiment, but the "true" V_{max} cannot be less than 85 nmol/mg protein/min, the rate of conversion of glucose under optimal conditions. The variation in the K_m values can be understood as being caused by the biphasic uptake kinetics of concentration dependence that result from the interaction of the facilitated diffusion carrier and the subsequent metabolism converting the glucose taken up and thus trapping it. The rate of uptake is governed by the combined kinetics of the transport and the enzymatic steps combined according to (Ter Kuile and Cook, 1994).

$$V = 1/2\left[\left(\frac{V_{max}(KR_{00} + R_{12}S) + S + K_m}{KR_{00} + R_{12}S - K_m(R_{21} + R_{ee}S/K)}\right) - \sqrt{\left[\left(\frac{V_{max}(KR_{00} + R_{12}S) + S + K_m}{KR_{00} + R_{12}S - K_m(R_{21} + R_{ee}S/K)}\right)^2 - 4\frac{V_{max}S}{KR_{00} + R_{12}S - K_m(R_{21} + R_{ee}S/K)}\right]}\right]$$

Curves of this type are at the lower concentrations primarily governed by the kinetics of the transporter, at the highest concentrations primarily by the V_{max} of the enzymatic reaction, and in the transition phase the control of the overall rate is shared by both steps, as predicted by the metabolic flux control theory. When data of such a system are plotted in a double reciprocal plot, the reported K_m will be smaller than the "actual" value of the transporter only. The reason for this is that at higher concentrations the V_{max} of the overall uptake process is lower than it would have been were only the transporter rate-limiting. Nonphosphorylated analogues, such as 6-deoxy-D-glucose, indeed have K_m's (1.54 mM, Eisenthal *et al.*, 1989) in the same range as the K_m for D-glucose determined by modelling using the above equation (1.96 mM). In addition, the apparent K_m can be increased by a diffusion gradient that may exist in the unstirred layer around the cell (Thomson and Dietschy, 1984). The actual concentration near the plasma membrane is then lower than in the measured concentration in the medium. The carrier is asymmetric by a factor of 1.8 (Eisenthal *et al.*, 1989), indicating that the K_m for influx equals 0.56 times the K_m for the efflux. This asymmetry is constitutive, in contrast to the asymmetry of the human erythrocyte carrier, which is asymmetric by a factor of 10 when phosphorylated but symmetric when unphosphorylated (Carruthers, 1990). These experimental data are confirmed by sequence information. Comparison of the gene of the *T. brucei* transporter with that coming from mammalian carriers shows that, while all other regions coding for parts thought to the essential are conserved, the phosphorylation site is missing in the *T. brucei* gene (Bringaud and Baltz, 1992).

While the mechanism for glucose transport across the plasma membrane of LS *T. brucei* seems well established, this is not the case for *T. brucei* insect form and *Leishmania* species. Two reports conclude that the *T. brucei* insect form accumulates glucose against the concentration gradient, based on the uptake of 2-deoxy-D-glucose (2-DOG) (Parsons and Nielsen, 1990; Munoz-Antonia *et al.*, 1991). Though these authors did take the phosphorylation of 2-DOG into account, they were not aware that the product formed is more readily hydrolyzed than glucose-6-phosphate. When D-glucose rather than an analogue was used, the biphasic dependence of glucose uptake on the external glucose concentration that is diagnostic for facilitated diffusion followed by enzymatic conversion was observed (Ter Kuile and Opperdoes, 1992a). In chemostat studies the internal concentration of free, unphosphorylated glucose never exceeded the external concentration (Ter Kuile and Opperdoes, 1992b). Considerable biochemical evidence exists indicating proton/glucose import as the mechanism for glucose transport in *L. donovani* (Zilberstein and Dwyer, 1984, 1985). Part of this work is based on the use of 2-DOG and is thus subject to the same criticism as above, but in addition the influx of glucose due to an artificially imposed concentration gradient caused an influx of protons. This flow of protons, however, was only 0.1–0.2% of the flow to be expected if the glucose/proton stoichiometry were 1 : 1 (Ter Kuile, 1993). In contrast to this active uptake, a (facilitated) diffusion component was found for the uptake of D-glucose by *L. donovani* (Ter Kuile and Opperdoes, 1993). The overall uptake of glucose was governed by the equation for equilibration

of the internal and external concentrations in addition to an enzymatic component, probably representing the metabolic conversion (Ter Kuile and Opperdoes, 1992a, 1993). From a theoretical point it is unlikely that a facilitated diffusion transporter and an active transporter would operate in parallel in the membrane of one compartment. All substrates accumulated by active transport would exit the cell through the facilitated diffusion carrier once the internal concentration exceeds the external. Thus, the physiological evidence would suggest facilitated diffusion as the mechanism for glucose uptake across the plasma membrane.

In recent years considerable information at the molecular level has become available on the *Leishmania* and *T. brucei* hexose transporters. The gene for a putative hexose transporter was first found in *L. enrietti* (Cairns *et al.*, 1989). The mRNA from this gene accumulated to a much higher level in the promastigote (insect) stage than in the amastigote form, which resides in mammalian macrophages (Cairns *et al.*, 1989). The gene is present in 8 copies that are tandemly clustered (Stein *et al.*, 1990). The first gene is transcribed to form a distinct stable RNA (Stack *et al.*, 1990). In the related species *L. donovani* two genes were cloned that encode for transporters belonging to the superfamily of facilitated diffusion glucose transporters (Langford *et al.*, 1992). One of these is expressed primarily in the insect stage and resembles strongly the transporter of *L. enrietti*, while the other is structurally quite different and is expressed constitutively. Similarly, two genes encoding hexose transporters were found in *T. brucei* as well (Bringaud and Baltz, 1993). One, THT1, is expressed in the bloodstream form, the other, THT2, in the insect form. The sequence homology is 80%. Even though all evidence strongly indicates that the cloned genes code for a facilitated diffusion carrier, the evidence at the functional level was lacking until the recent study by Bringaud and Baltz (1993), expressing the THT1 gene in *Xenopus leavis* oocytes. The RNA was injected in *X. leavis* oocytes and caused a dramatic increase of the ability of the oocytes to take up 2-DOG. The effects of inhibitors and competing hexoses were very similar to the effects of the same compounds on 2-DOG uptake by *T. brucei* bloodstream forms. This evidence can be considered sufficient to conclude that the THT1 gene codes for the facilitated diffusion hexose carrier of the LS bloodstream form of *T. brucei*.

When we combine all information available on glucose transport in trypanosomatidae, the following picture emerges: It is beyond dispute that glucose is transported across the plasma membrane of the *T. brucei* bloodstream form by a facilitated diffusion carrier. The evidence for active uptake as the mechanism for the inset stage is not sufficiently strong and contradicted by physiological studies using D-glucose and possibly also by the molecular data showing that the THT2 gene has 80% sequence homology with the THT1 gene, a proven facilitated diffusion carrier. Data on the transport of glucose and glycolytic intermediates across glycosomal membranes, of any species, are sorely lacking. The question of active transport versus facilitated diffusion is the subject of even more conflicting evidence in *L. donovani*. As discussed above, the biochemical and the physiological evidence contradict each other, while the homology of the hexose transporter gene with the family of facilitated diffusion carriers is not sufficient to decide the issue. Interestingly, one of the three genes for hexose transporter found in *L. donovani* is sufficiently different from the other two that it could encode a mechanistically different carrier, possibly a H^+/hexose symporter. Because it is constitutively expressed, it would be a plausible candidate for a transporter located in the membrane of an organelle. Such a scheme, facilitated diffusion across the plasma membrane and active transport probably across the glycosomal membrane, would account for the smaller than expected flow of electrons upon addition of glucose to deenergized cells and thus reconcile both views. At present this is nothing but speculation to be amended, rejected, or confirmed by future research. In particular, the expression of the RNA of these genes in *X. leavis* oocytes, as was done so elegantly with the RNA of the *T. brucei* hexose transporter, will in all likelihood shed a decisive new light on these issues. After the function and mechanisms of each of the transporters involved have been determined, the exact location in the cell will have to be established.

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