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pH Regulation in Glycosomes of Procyclic Form *Trypanosoma brucei**

Sheng Lin[a], Charles Voyton[a,c], Meredith T. Morris[b], P. Christine Ackroyd[c], James C. Morris[b], and Kenneth A. Christensen[c]

[a]Department of Chemistry and [b]Department of Genetics and Biochemistry, Clemson University, Clemson, SC 29634, [c]Department of Chemistry and Biochemistry, Brigham Young University, Provo, UT 84602

Running title: *pH Regulation in Glycosomes of Procyclic Form T. brucei*

To whom correspondence should be addressed: Kenneth A. Christensen, Department of Chemistry and Biochemistry, Brigham Young University, Email: kenc@chem.byu.edu; Tel: 801-422-0249.

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ABSTRACT

Here we report the use of a fluorescein-tagged peroxisomal targeting sequence peptides (F-PTS1, acetyl-CKGGAKL) for investigating pH regulation of glycosomes in live procyclic form *Trypanosoma brucei*. When added to cells, this fluorescent peptide is internalized within vesicular structures, including glycosomes, and can be visualized after 30-60 min. Using F-PTS1 we are able to observe the pH conditions inside glycosomes in response to starvation conditions. Previous studies have shown that in the absence of glucose, the glycosome exhibits mild acidification from pH 7.4 ± 0.2 to 6.8 ± 0.2. Our results suggest that this response occurs under proline starvation as well. This pH regulation is found to be independent from cytosolic pH and requires a source of Na^+ ions. Glycosomes were also observed to be more resistant to external pH changes than the cytosol; placement of cells in acidic buffers (pH 5) reduced the pH of the cytosol by 0.8 ± 0.1 pH units, whereas glycosomal pH decreases by 0.5 ± 0.1 pH units. This observation suggests that regulation of glycosomal pH is different and independent from cytosolic pH regulation. Furthermore, pH regulation is likely to work by an active process, since cells depleted of ATP with 2-deoxyglucose and sodium azide were unable to properly regulate pH. Finally, inhibitor studies with bafilomycin and EIPA suggest that both V-ATPases and Na^+/H^+ exchangers are required for glycosomal pH regulation.

Trypanosomatids are unicellular parasitic organisms of the class Kinetoplastida. It is estimated that members of the disease-causing trypanosomatids, including *T. brucei*, *T. cruzi*, and *Leishmania spp.* infect over 30 million people a year (1). The prevalence of these parasite infections is exacerbated by the difficulties in developing effective therapeutic agents as well as the social, geopolitical, and impoverished conditions of areas typically impacted by these pathogens.

Since trypanosomes travel between insect vectors and mammalian hosts, the ability to regulate metabolism under a wide range of conditions is essential for parasite survival. For example, *T. brucei* residing in the tsetse fly (the procyclic form, PF) can metabolize both glucose and amino acids (primarily proline), whereas only glucose is used while in the mammalian host (2). Compartmentalization of glycolytic enzymes within the glycosome has been suggested as the primary means of regulating glycolysis (3, 4). In addition to compartmentalization, glycosomal pH has been shown to affect or modulate activity of key glycolytic enzymes (2).

We have previously shown that PF parasites acidify the glycosomal compartment in response to glucose deprivation (2, 5). Since fatty

acid, purine, polyamine, and amino acid metabolism are highly pH dependent, glycosomal acidification under low glucose conditions suggests that *T. brucei* may be regulating glycosome pH in order to inactivate certain metabolic processes while simultaneously activating alternate potentially more beneficial processes (6). However, the mechanism of glycosomal pH regulation is unknown. In most higher eukaryotic cells, pH regulation is partially maintained by exchange of metabolically generated protons for $Na^{+,}$ using Na^+/H^+ antiporters (7). This regulatory process is driven by a Na^+ gradient created by Na^+/K^+ ATPases. At low external pH, Na^+/H^+ exchange is unfavorable; as a result, cells typically also utilize ATP-driven proton pump(s) for pH maintenance. Previous studies performed with inhibitors and genetic analysis of proton pumps in PF *T. brucei* have suggested involvement of both Na^+/K^+ ATPases and ATP-driven proton pumps. For example, studies of cytosolic pH regulation suggest that cytosolic pH is regulated by plasma membrane H^+-ATPase (8, 9). Studies on *T. brucei* lysosomes and acidocalcisomes (intracellular acidic vesicles containing polyphosphates) have shown that pH regulation requires a combination of various vacuolar H^+-ATPases (V-ATPase) (10, 11) and Na^+/H^+ exchangers (NHE)(12); glycosomes may regulate pH similarly. While studies on peroxisomes, a mammalian organelle that is the closest evolutionary relative to glycosomes, suggest that only F-Type ATPases are present (13), other studies have shown that V-ATPases indirectly contribute to peroxisome regulation by acidification of the cytosol (14). Furthermore, proteomic analysis has identified the presence of putative genes that encode V-ATPases in the glycosome (15) of *T. brucei*. Hence, both V-ATPases and NHEs are strong candidates for glycosomal pH regulation.

PTS1 sequences are C-terminal tripeptides that have also been used to traffic cargo to the peroxisomal lumen of yeast, plants, insects, and mammalians cells (14, 16–18). We have previously demonstrated that conjugating fluorescein to PTS1 delivers the ratiometric pH sensor to glycosomes for quantitative measurements of pH *in situ* (5). Using this approach, we demonstrate the effect of proline and glucose starvation on glycosomal pH in PF *T. brucei* as well as the effects of incubation in acidic buffers (pH_e). We also determined that glycosomal pH is actively regulated and is independent of cytosolic pH. This regulation process is ion and ATP dependent, and is likely regulated by a combination of glycosomal V-ATPases and Na^+/H^+ exchangers.

Results

Glycosomal pH Response to Nutrient Deprivation. PF trypanosomes can adapt their metabolism to conditions of low glucose through the up-regulation of amino acid metabolism (19, 20). We have previously quantified the glycosomal pH response to glucose deprivation (5). Here we compare glycosomal pH response in the presence or absence of both glucose and proline, an alternate amino acid carbon source metabolized by PF *T. brucei*. To measure intraglycosomal pH, we loaded live cells with F-PTS1 under various nutrient deprivation conditions, and measured the pH-dependent spectral change in fluorescein emission ratio followed by intracellular pH calibration with digitonin in CB. Representative images and calibration curves are shown in Figure S1A and B. Similar measurements and calibration were performed using the fluorescent dye BCECF, which localizes to the cytosol rather than the glycosome (Figure S1A and B).

Figure 1A shows the change in glycosomal pH over 120 min after depriving the cells of nutrients following preconditioning with glucose, proline, or both glucose and proline. Consistent with our previous observations, cells conditioned to glucose underwent rapid glycosomal acidification from 7.6 ± 0.2 to 6.8 ± 0.2 within 15 minutes (5). However, when cells conditioned to proline-supplemented buffers (either proline alone or a mixture of proline and glucose), glycosomal pH remained constant (pH 7.4 ± 0.1) over the same 15 min period. Removal of the nutrients from cells conditioned to proline had no short-term effect on intraglycosomal pH. Instead, cells conditioned to proline undergo a much slower acidification.

In Figure 1B we also measured glycosomal pH in trypanosomes which were reintroduced to nutrients following a prolonged period of starvation (2 h). Reintroduction of glucose (or a combination of glucose and proline) results in alkalization back to physiological pH 7.5 over 20 min. Hence, the time periods required for acidification and alkalization in response to glucose availability are similar. However, re-alkalization in the presence of proline is much slower, requiring nearly 40 min to reach normal physiological pH.

Together, these results suggest that the glycosomal pH response to proline is markedly different than the response to glucose. Acidification and alkalization in response to proline deprivation and supplementation is 8 and 2 times longer, respectively, than for glucose. The difference in glycosomal pH response to the different nutrient sources suggests a difference in regulation initiated by the respective nutrient sources. Mechanistic differences in nutrient response are expected, since proline is transported to the mitochondria for oxidative phosphorylation (21), a process that bypasses the glycosomes, while glucose metabolism occurs solely in glycosomes.

Glycosomal and Cytosolic pH Response to Acidic External pH. Due to marked changes in the external environments during the trypanosome life cycle, trypanosomes must contain robust mechanisms for quickly adapting to a variety of conditions. For example, PF *T. brucei* replicating in the alimentary tract of the tsetse fly are exposed to shifts in pH up to 3 pH units during blood meals (22). PF trypanosomes must also maintain appropriate internal cytosolic pH values in the presence of a range of external pH values; previous studies have determined the cytosolic pH of PF *T. brucei* to be approximately 7.6, a value that is maintained in the presence of an external pH (pH_e) range of 6-8 (8). However, measurements of glycosomal pH as a function of external pH have not previously been performed, despite the known pH-sensitivity of glycolytic enzyme function. In order to compare regulation of glycosomal and cytosolic pH, we monitored both glycosomal and cytosolic pH in cells exposed to acidic external buffers at pH_e 5.0, 6.0 and 7.4, over a period of 60 minutes. Glycosomal pH measurements were carried out as above; while cytosolic pH measurements were carried out using the pH-sensitive ratiometric dye (in this case, BCECF), loaded into the cytosol; intracellular calibration of the BCECF 495/440 emission ratio is shown in Figure S1 B (dotted line). A comparison of pH values obtained from ratiometric measurements in the cytosol and glycosomes, as a function of varied external pH, is presented in Figure 2A. These data demonstrate that acidic pH_e results in greater acidification of cytosolic pH compared to the glycosomal pH. At external pH_e 6, cytosolic pH decreased from 7.3 ± 0.1 to 6.9 ± 0.1, a response consistent with previous studies (8), while glycosomal pH changed from 7.4 ± 0.2 to 7.1 ± 0.1. Similarly, at pH_e 5, cytosolic pH decreased to 6.5 ± 0.1 compared to the glycosomes that acidified to pH 6.8 ± 0.1. Figure 2B shows a time course of the cytosol and glycosome pH change as a response to acidic pH_e. These data demonstrate that most of the acidification in the cytosol occurs within 10 minutes of buffer exchange into acidic pH_e whereas glycosomal pH decreased gradually over time. Together, these data suggest that the two compartments are not in equilibrium with each other, and that regulation of glycosomal pH may be independent from cytosolic pH.

To investigate the ability of glycosomes to maintain a pH independent from the cytosol, we titrated varying amounts of digitonin to cells equilibrated at pH_e 5 under conditions of nutrient deprivation (no glucose and no proline). Previous studies determined that a minimum of 500 µg/mL digitonin is required to compromise the glycosomal membrane (23) while lower digitonin concentrations act on the plasma membrane alone. By titrating the detergent and monitoring the pH of both compartments, we assessed the ability of glycosomes to maintain a pH independent of cytosolic pH (Figure 2C). As with previous measurements, glycosomal pH was quantified using F-PTS1 while cytosolic pH was quantified using BCECF. The addition of 10 µg/mL digitonin was sufficient to permeabilize the cellular membrane to protons and resulted in a cytosolic pH decrease from 6.5 ± 0.1 to 5.7 ± 0.1; additional

digitonin further acidified the cytosol as it became equilibrated with pH_e. In contrast, glycosome pH was maintained at a pH approximately 0.8 pH units higher than the cytosol following treatments with 10 µg/mL digitonin, as the detergent was not at a sufficient concentration to compromise the membranes of the organelle. Only after the addition of 500 µg/mL digitonin (i.e. after glycosomal membrane permeabilization) did the glycosomal pH equilibrate with cytosolic pH. The resistance of glycosomal pH to changes in cytosolic pH in the absence of glycosomal membrane permeabilization supports the hypothesis that glycosomes regulate internal pH, likely a consequence of different pH requirements in each compartment.

Effects of Na^+ and K^+ on pH regulation. Most cells utilize a series of active or passive ion transporters, mainly those involving transport of Na^+ and K^+, to regulate pH. To investigate the possible contribution of the different ion transporters to pH control in *T. brucei*, we compared the maintenance of steady state pH in the cytosol and glycosomes in the presence and absence of these ions. PF *T. brucei* were equilibrated at pH_e 5.0 in Na^+-free or K^+-free buffers. The pH was monitored over 60 minutes, using BCECF as the cytosolic pH probe and F-PTS1 as the glycosomal pH probe. As shown in Figure 3A, cytosolic pH control was observed to be more dependent on a K^+ source than Na^+. Absence of K^+ from the buffer resulted in a decrease of cytosolic pH from 6.3 ± 0.2 to 5.6 ± 0.2 at pH_e 5.0. (Figure 3A). However, glycosomal pH control was more dependent on Na^+ (Figure 3B). Upon removal of Na^+, glycosomal pH decreased from 6.6 ± 0.1 to 5.3 ± 0.3. This observation suggests that the cell's ability to regulate steady-state pH in the cytosol depends on K^+, while glycosomal pH regulation requires Na^+.

We next determined whether the absence of K^+ or Na^+ affected glycosomal acidification under starvation conditions. Cells were incubated in nutrient-free mPBS without either Na^+ or K^+ for 1 hour at physiological pH (Figure 3C). Cells in mPBS containing only Na^+ exhibited the previously observed acidification response to starvation conditions - a decrease of 0.6 to 0.8 pH units. However, cells incubated in mPBS containing only K^+ were unable to acidify their glycosomes under the same conditions. Instead, the glycosomal pH was similar to cells incubated in mPBS supplemented with glucose. Differences in the glycosomal pH response of the cells incubated in Na^+ and K^+ buffers were found to be statistically different by ANOVA. This reliance of pH control on different ions for the respective compartments suggests that different membrane transporters are responsible for regulation in the cytosol and glycosome. This may be necessary for the parasite to alter the pH of its glycosomes independently to maintain appropriate glycolytic enzyme function, even in the presence of shifting cytosolic pH.

Effects of ATP on pH regulation in glycosomes. Previous studies on pH regulation in mammalian peroxisomes have shown that this process is ATP dependent (14). Because we have observed the ability of *T. brucei* glycosomes to maintain a more alkaline pH than the cytosol or external environment, an active ATP-driven process is likely operating in these kinetoplastid organelles as well. Under normal starvation conditions with nutrient-free buffer, complete ATP-depletion in the parasites occurs very slowly, and no change in the ability to regulate glycosomal pH during incubation in acidic pH_e could be detected. In order to better study the role of ATP in regulating glycosomal pH, we employed a method that uses 2-deoxyglucose (2DG) and sodium azide (NaN_3) to deplete the cells of ATP. 2DG is converted into 2-deoxyglucose-6-phosphate (2D6P) by hexokinase and the consumption of ATP. Normally, this investment of ATP results in the generation of ATP during later stages of the glycolysis cycle. However, unlike conversion from glucose, conversion to 2D6P inhibits further ATP production and thus actively depletes the cell of ATP. At 100 mM 2DG, cells can be depleted of up to 90% ATP in minutes (24). 2DG alone does not entirely deplete the cells of ATP; besides glycolysis, oxidative phosphorylation is also responsible for ATP generation. To address this residual ATP formation we also used NaN_3, an inhibitor of oxidative phosphorylation in mitochondria, to

deplete remaining ATP. 2DG and NaN_3 have been used together in mammalian cells for ATP depletion studies and have shown to adequately deplete ATP for up to 1 hour without compromising cell viability (25–27).

We first determined the optimum concentration of 2DG/NaN_3 needed for *T. brucei* ATP depletion. Cells were equilibrated under starvation conditions in PBS for 1 hour, after which various concentrations of 2DG/NaN_3 were added and cellular ATP concentration was measured after 10 minutes by a luciferase assay. The cells also were allowed to recover after reintroduction of nutrients via the supplementation of culture media. 10 min after nutrient reintroduction, the ATP levels were again measured (Figure 4A). Normal starvation conditions were observed to decrease basal ATP levels to ~30% of that for untreated cells cultured in media (dotted horizontal line). However, addition of 2DG/NaN_3 further decreased ATP levels to ~18% at 1 mM and as low as ~9% at >10 mM. Hence, addition of 2DG/NaN3 to cells already under starvation conditions resulted in a further three-fold depletion of ATP. Additionally, we demonstrated that cells treated with 2DG/NaN3 were able to regenerate ATP upon supplementation of carbon sources. In cells depleted with <10 mM 2DG/NaN_3, ATP recovery reached ~78% of untreated cell ATP levels, similar to cells depleted from starvation alone. The inability these ATP-depleted cells to return to full ATP levels upon subsequent nutrient supplementation is likely due to some cell death during starvation, an observation consistent with previous studies of ATP depletion in mammalian cells (27). Higher treatments of 2DG/NaN_3 resulted in lower recovery after nutrient supplementation, down to only ~60% recovery at 50 mM. From these results, we determined that 10 mM of 2DG/NaN_3 provided optimum ATP depletion with minimal loss of cell viability. A comparison of cell viability (Figure S2) between nutrient-free PBS with and without 10 mM 2DG/NaN_3 over 60 minutes showed no significant difference in viability over the first 30 minutes, and only ~5% less over longer periods of time.

A time course of the ATP depletion rate and ATP replenishment using 2DG/NaN_3 was performed on cells grown in normal culture conditions (Figure 4B). ATP levels in untreated cells were measured before exchange into nutrient-free PBS containing 10 mM 2DG/NaN_3, then ATP levels were monitored over time as cells responded to ATP deletion. In this case, cells reached maximum ATP depletion of ~90% in 8-10 minutes. When ATP-depleted cells were treated with nutrients to allow ATP levels to be replenished, regeneration back to starting levels occurred in less than 4 minutes.

To gain a better understanding of the cell's steady-state ATP levels, we also compared the ATP levels in *T. brucei* under various inhibitor and nutrient conditions (Figure 4C). Cells incubated in PBS resulted in a 3-fold decrease in ATP levels. However, cells incubated in PBS plus 10 mM 2DG/NaN_3 showed a 12-fold decrease in ATP levels compared to media control, and were able to recover 80-90% of their starting ATP upon nutrient reintroduction. Interestingly, cells incubated with proline alone as the carbon source maintained ATP levels similar to that for cells cultured in growth media, while cells in buffer containing glucose or both glucose and proline together contained slightly less total ATP. This observation may reflect *T. brucei*'s preference for glucose over proline as a carbon source (28), and the lower efficiency of ATP generation from glucose (21).

Using this method of ATP depletion, we were able to study PF trypanosomes' ability to regulate glycosomal pH under conditions of very low ATP. We first assessed whether ATP was necessary for maintaining steady-state glycosomal pH under acidic pH_e. PF *T. brucei* in pH_e 7.4 were depleted of ATP, followed by a buffer exchange into pH_e 6.0, and glycosomal pH was observed throughout using F-PTS1. A time course of glycosomal pH under these conditions is shown compared to that for non-ATP depleted cells in Figure 5A. These data show a significant drop in glycosomal pH in ATP-depleted cells (from 7.6 ± 0.2 to 6.6 ± 0.2) over a period of 15 minutes after buffer exchange into pH_e 6.0. Following the relatively rapid drop, glycosomal pH remains static at ~ 6.6. In comparison, non-ATP depleted cells under the same conditions show only a slight drop

in pH (from 7.7 ± 0.2 to 7.4 ± 0.2). Together, these data suggest that ATP is involved in control of glycosomal pH.

We compared the effects of ATP depletion on glycosomal pH to that of cytosolic pH (Figure 5B). BCECF and F-PTS1 were used to quantify cytosolic and glycosomal pH 1 hour after ATP depletion. As expected, ATP depletion results in no significant change of pH in either compartment at external physiological pH. At pH_e 6.0, cytosolic pH in ATP-depleted and non-depleted cells are indistinguishable; both treated and untreated cells resulted in a cytoplasmic pH of ~6.6. In comparison, the removal of ATP has a much greater effect on pH control in the glycosome than in the cytosol; ATP depletion decreased glycosomal pH by ~0.6 in acidic pH_e. Significantly, the glycosomal pH under ATP-depletion seems to be in equilibrium with the cytosolic pH. These observations show that ATP depletion does not have a significant impact on cytosolic pH regulation under acidic pH_e, but does affect glycosomal pH regulation. Without ATP, glycosomal pH regulation becomes inactive, and the pH equilibrates with cytoplasmic pH.

We next examined the effect of ATP depletion on regulating glycosomal acidification. As shown previously (Figure 2A), *T. brucei* are able to reversibly acidify their glycosomes by 0.6 to 0.8 pH units in response to nutrient deprivation. To determine whether this process is ATP dependent, we incubated cells in glucose-free buffer under ATP depletion conditions and monitored glycosomal pH by F-PTS1 analysis (Figure 6). Consistent with previous observations, cells under starvation conditions undergo glycosomal acidification over 20 minutes, then remaining constant at pH ~6.8. In cells incubated under starvation conditions in the presence 2DG/NaN_3, a similar acidification response is observed initially (during the first 20 minutes after glucose removal), but is subsequently followed by alkalinization back to ~7.4. Presumably, proper functioning of the glycosomal pH acidification process is prevented after the basal supply of ATP is exhausted (≤20 minutes after nutrient depletion) in the presence of 2DG/NaN_3. This observation is consistent with our previous data indicating that maximum ATP depletion using this method requires a minimum of 10 minutes (Figure 4B).

Effect of protein inhibitors on glycosomal pH regulation. Previous studies of trypanosome subcellular compartments have shown that Vacuolar H^+-ATPases (V-ATPase), phosphorylation P-type ATPases (P-ATPase) and Na^+/H^+ exchangers (NHE) are responsible for pH regulation in acidocalcisomes, lysosomes, and the cytosol (9–11, 29–31), respectively. Our data indicate that glycosomal pH regulation requires ATP and Na^+ ions, and is therefore consistent with regulation by ATPases and/or NHE. NHEs are carrier-mediated electroneutral exchangers of Na^+ for H^+ and do not directly consume metabolic energy. However, NHEs require the presence of phosphatidylinositol 4,5-bisphosphate (PIP2), and acute depletion of ATP has been shown to result in dephosphorylation of PIP2 and a large decrease in NHE activity (32). As a result, involvement of either ATPase or NHE proteins in glycosomal pH regulation would be reflected in reduced pH regulation at low ATP concentrations. In addition, each of these transporters have been identified as putative *T. brucei* glycosomal proteins by genetic analysis (33).

To determine whether these proteins are involved in glycosomal pH regulation, we examined the effect of V-ATPase, P-ATPase, and NHE inhibitors on glycosomal pH. We treated cells with bafilomycin, which inhibits V-ATPase, and vanadate, which inhibits P-ATPase, to assess the role of these integral transmembrane proteins in observed glycosomal pH regulation. Bafilomycin is widely applied in mammalian systems (34), and has been used to determine the role of V-ATPases in regulating lysosomal pH in *T. brucei* (10) and cytosolic pH in *T. cruzi* (35). Vanadate has been successfully utilized to study the properties of P-ATPases in kinetoplastids including *T. brucei*, *T. cruzi*, and *Leishmania donovani* (9, 31). We also treated cells with 5-(N-ethyl)-N-isopropylamiloride (EIPA), a protonophore inhibitor of NHE that has been previously used in studies of *T. cruzi* and *T. brucei* cytosolic pH regulation (8, 35). For these inhibition experiments, PF *T. brucei* were incubated in nutrient-free mPBS with bafilomycin

(2 µM), vanadate (500 µM), or EIPA (100 µM); cells were treated with each inhibitor by itself and in combination, and glycosomal pH was quantified with F-PTS1. The resulting glycosomal pH data were compared to that for trypanosomes both without inhibitors and with nutrient supplementation (Figure 7A). We note that while addition of inhibitors was observed to decrease cell viability by ~30-40% by Cell Titer Blue assay (Figure S3), much of this effect (~20%) results from the vehicle solvent (DMSO) in which the inhibitors are dissolved. Vanadate does not require DMSO for solubility and is dissolved in H_2O; cells incubated with vanadate showed substantially higher viability. To minimize the impact of inhibitor effects on viability, dead cells were identified by morphology and motility during pH analysis and excluded from calculations. As a result, we report pH values from the glycosomes of only viable cells.

Addition of bafilomycin alone resulted in a very modest alkalinization of 0.1 pH units, while vanadate and EIPA by themselves resulted in no statistical change in glycosomal pH. However, cells incubated with both bafilomycin and EIPA showed a significant pH increase by ANOVA (~0.4 pH units) compared to normal starvation conditions without inhibitors (nutrient-free mPBS), suggesting that the glycosomal acidification process is unable to occur. While cells incubated with all three inhibitors showed the same increase, vanadate by itself as well as when added to one of the other inhibitors showed no change in glycosomal pH and it is likely that vanadate has no effect on glycosomal pH regulation. Therefore, these inhibition results suggest that a combination of V-ATPases and NHEs are responsible for glycosomal acidification.

To further assess the effect of inhibitors on glycosomal acidification, a time-course analysis of glycosome pH during treatment with bafilomycin and EIPA is shown in Figure 7B. PF *T. brucei* were first incubated under starvation conditions (i.e. in nutrient-free mPBS), followed by the addition of bafilomycin and EIPA (vertical dotted line) while monitoring glycosomal pH with F-PTS1 over 30 minutes. A significant alkalization occurred during the first 15 minutes (glycosomal pH increases by 0.4 pH units), but was followed by reversion back to non-starvation pH levels. This rate of glycosomal pH increase by inhibition was similar to the natural alkalization rate for cells that were reintroduced to metabolic carbon sources, as previously reported (5), suggesting that T. *brucei* may control alkalization by inactivating glycosomal V-ATPases and NHEs rather than by activating other integral proteins.

Discussion

T. brucei must be able to adapt to constantly changing environments as the nutrient composition changes drastically during a blood meal or when transitioning to a mammalian host. These studies of cytosolic and glycosomal pH strongly suggest that T. *brucei* actively regulate subcellular pH in response to different environmental conditions. Cells incubated in the presence of glucose or proline as the primary nutrient source exhibited glycosomal acidification after nutrient removal. The observed acidification rate was 8x slower upon proline removal than upon glucose removal. Differences in acidification rates are likely due to differences in the respective metabolic pathways. Proline can be converted to glucose-6-phosphate through gluconeogenesis allowing glucose-dependent pathways, such as the phosphate-pentose pathway, to operate in the absence of glucose (36). This alternate pathway may explain why the proline acidification response is similar to glucose starvation, albeit occurring at slower rates.

Glycosomes, which more effectively maintain lumenal pH at pH_e between 6 and 7 is significantly different than that observed for the cytosol. This is consistent with previous studies in *T. brucei*, which suggest that cytosolic pH control occurs through a passive mechanism (8). Cytosolic pH regulation is compromised at pH < 6.0, due to increased proton influx. We note that trypanosomes under physiological conditions typically do not encounter such acidic environments and cytosolic responses to these extreme conditions are therefore physiologically unnecessary. When glycosomes were examined under similarly acidic external conditions, it was evident that these organelles

utilize a more robust mechanism for regulating pH. Even under incubation buffer at pH_e 5.0, glycosomes were able to maintain a pH slightly below 7. This effect was observed in the presence of selective permeabilization and equilibration of the cytosol to pH_e 5. In this case, the glycosomal pH was maintained at 1 pH unit higher than the surrounding cytosol. This observation suggests that the glycosomal membrane is able to counteract the cytosolic pH and independently regulate its own pH. Independent glycosomal pH regulation may be physiologically necessary given the pH-dependence on activity of many glycolytic proteins (37, 38). For example, the glycosomal enzyme *T. brucei* hexokinase (TbHK) is responsible for converting glucose to glucose-6-phosphate in the glycolysis pathway, and has been shown to be inactivated at pH 6.5 (2).

Previous studies on subcellular pH regulation in kinetoplastid and eukaryotic species suggests a strong dependence on ion and ATP availability for responses to pH changes (11–14, 39). We have shown that incubation of *T. brucei* in buffer lacking Na^+ leads to poorly regulated glycosomal pH when compared to parasites in the presence of Na^+. Without Na^+, the steady-state pH of the glycosome is not as tightly maintained under acidic pH_e and the acidification effect observed under starvation conditions no longer occurs. Similarly, we have demonstrated that ATP is also necessary for proper pH regulation. Cells under normal starvation conditions maintain a small basal level of ATP sufficient to counteract an acidic pH_e 5.0. However, when cells were treated with 2DG and NaN_3 to more fully deplete ATP, the glycosome did not resist acidification and glycosomal pH reached equilibrium with cytosolic pH. Additionally, cells under ATP depletion and starvation were unable to induce an acidification process after 20 minutes, presumably when the cell's basal ATP storage had been depleted. This is similar to the effects of glucose deprivation on V-ATPase observed in other eukaryotic species, such as yeast. When yeast are deprived of glucose, their basal ATP concentration decreases to less than 10% of their normal values (40). Low ATP levels combined with other cellular factors leads to the inhibition of V-ATPase through protein disassembly (41). We show here that unlike yeast, V-ATPase in PF trypanosomes still function during glucose deprivation, possibly due to the greater ATP content (~30%) in these cells. Due to the importance of glycosomal pH regulation in trypanosomes, these parasites may have developed divergent pathways that prioritize the necessity for glycosomal acidification during starvation. It is interesting to note that under starvation conditions, *T. brucei* continue to expend their remaining ATP to maintain glycosomal pH, an observation that emphasizes the importance of this organelle for cell survival. Only when cells are more fully depleted of ATP (treatment with 2DG and NaN_3) does glycosomal acidification no longer occur.

To determine the proteins responsible for pH regulation, we tested various inhibitors for NHE (EIPA), V-ATPase (bafilomycin), and P-ATPase (vanadate) for effects on glycosomal pH acidification in response to starvation conditions. None of the inhibitors had significant effects ($p < 0.001$) in isolation. However the combination of EIPA and bafilomycin prevented the expected acidification response, suggesting that both NHE and V-ATPases are required for glycosomal pH regulation. Since neither of these proteins alone is enough to maintain normal function, requirement for dual transport proteins may be a redundant mechanism for more robust response to possible environmental changes. An analysis of the recovery rate from acidification after the introduction of inhibitors reveals a rate of alkalization similar to the rate that occurs upon reintroduction of glucose after a period of starvation. This observation suggests that the glycosome may revert back to normal pH not by the activation of alternate protein channels, but by inactivating the ones responsible for acidification. These results are different from other eukaryotic species which rely on a combination of peroxisomal F-type ATPase and membrane permeability to regulate pH. More studies must be done to fully understand the glycosomal pH regulation process. However, our results strongly suggest that NHE and V-ATPases are involved in glycosomal pH regulation. While the experiments presented were performed with the PF insect stage

of the parasite, the mammalian BF stage exhibits an even greater reliance on glucose, as it is the sole metabolite used for ATP generation. Both forms of the parasite utilize similar pH-dependent enzymes (e.g. hexokinase), and may require similar pH regulation mechanisms. Further investigation of these proteins and its role in the infectious BF stage may lead to potential therapeutics that operate by selectively interfering with the trypanosome's ability to regulate glycosomal pH.

EXPERIMENTAL PROCEDURES

Reagents and Buffers. Fluorescein-tagged peroxisomal transport sequence (F-PTS1, acetyl-C{K(FITC)}GGAKL; 1107.29 MW) was synthesized by Genscript. Bafilomycin, 5-(N-ethyl)-N-isopropylamiloride (EIPA), and ortho-vanadate inhibitors were purchased from LC Laboratories, Toronto Research Chemicals Inc., and EMD Millipore respectively. EIPA and Bafilomycin were dissolved in dimethyl sulfoxide (DMSO) at 100x working concentration before use. 2',7'-Bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein (BCECF) dye for cytosolic pH quantification was purchased from Life Technologies. 2-deoxy-D-glucose (2DG) and sodium azide (NaN_3) for ATP depletion studies were purchased from Alfa Aesar and BDH chemicals respectively. Luciferase assay for analyzing cell ATP content was purchased from Promega. Because growth media results in binding and aggregation of F-PTS1 probes, cell loading with the labeled peptide was carried out in serum-free buffers. Depending on the condition of interest, cells were incubated in Voorheis' modified PBS (mPBS; 137 mM NaCl, 3 mM KCl, 16 mM Na_2HPO_4, 3 mM KH_2PO_4, pH 7.6) (42) containing either 10 mM glucose, 10 mM proline, or no carbon sources for starvation analysis. For pH studies and internal calibration, cells were incubated in calibration buffer (CB; 130 mM KCl, 1 mM $MgCl_2$, 15 mM HEPES, 15 mM MES) adjusted to desired pH ranges from 4 to 8.

Microscopy. Images were captured by epifluorescence microscopy (Olympus IX71) with Xe lamp excitation. pH quantification of F-PTS1 and BCECF were measured by the intracellular 495/440 nm ratio after calibration with CB (pH 4-8) and digitonin as described previously (5). For ratiometric imaging of fluorescein-based probes (F-PTS1 and BCECF), single band excitation filters [440 nm (20 nm band-pass), 495 nm (10 nm band-pass)] and single band emission filters [535 nm (25 nm band-pass)] were used. An Orca-ER CCD (Hamamatsu) was used for image acquisition and control of all microscope components and all image processing was performed using Slidebook 5.0 (Intelligent Imaging Innovations). All data and statistical analysis acquired from ratiometric fluorescent micrographs were determined as follows: Fluorescent background signals were determined by average intensities of cell free regions at 495 and 440 nm and subtracted from each image. Each measurement point is a representation of cells from several fields taken within 1 min and binned together. Since some of the peptide may ultimately be delivered to the lysosomes, fluorescent compartments containing emission ratios consistent with lysosomal compartments (490 nm / 440 nm < 1.2; pH < 5.5) were excluded from analysis. Dead cells, as determined by morphological changes and lack of motility, were excluded from analysis. Measurements were collected at sample sizes of at least 25 cells and errors are reported as ± standard error of mean (SEM).

Cell response to nutrient deprivation, external pH (pH_e) changes, and Na^+/K^+ ions. PF *T. brucei brucei* strain 427 (29-13) were used in cellular studies. Unless noted, all studies involving F-PTS1 uptake in these cells was performed as described previously (5). Briefly, PF cells were grown in SDM-79 (43, 44) at densities between 5 x 10^5 and 5 x 10^7 cells/ml in 27.5°C and 5% CO_2. Cells were centrifuged and resuspended to a final concentration of 1 x 10^8 cells/ml in mPBS (for nutrient deprivation and ion analysis) or CB (for pH_e analysis) with either 90 µM F-PTS1 or 0.1 µM BCECF for 60 min (27.5ºC, 5% CO_2,). After probe uptake, cells were washed 3 times by centrifugation (750xg; 5 min) with incubation buffer and placed into a microscope perfusion chamber with fresh buffer. The perfusion chamber allows for quick buffer exchange while continuously monitoring

495/440 nm ratio for pH quantification. For glucose deprivation, cells were first incubated in mPBS containing 10 mM glucose, and replaced with glucose-free mPBS to simulate nutrient deprivation. Effects of acidic pH_e were monitored by buffer exchange from CB at pH 7.4 into CB set to either pH 6 or 5. Finally, studies on the effects of Na^+/K^+ were monitored by perfusion into mPBS lacking the respective ions. Measurements from 495 / 440 nm emission ratio were quantified by correlation to a pH titration curve with cells permeabilized by digitonin as mentioned previously.

Digitonin Titration. Cells were separately incubated with either F-PTS1 or BCECF in CB pH 7.4 with the standard uptake protocol as mentioned above. Cells were then combined together in a microscopic perfusion chamber such that each cell had either glycosomal or cytosolic labeling; combination of loaded cells is necessary as both probes have the same spectral properties and labeled glycosomes would not be distinguishable from labeled cytosol. The cells were then buffer exchanged into CB pH 5.0 and equilibrated for 15 min. Digitonin (10–500 µg/ml) was titrated at various intervals by buffer exchange into CB pH 5 containing the detergent at increasing concentrations. After each addition, the cells were equilibrated for 5 minutes before imaging at 495/440 nm for ratiometric quantification in both F-PTS1 and BCECF-loaded cells.

ATP deprivation and effect on pH regulation. To optimize ATP deprivation, 1 x 10^6 cells were pelleted (2 x 5 min, 750 x g) and resuspended in nutrient-free mPBS containing 1 to 50 mM 2-deoxyglucose (2DG) and Sodium Azide (NaN_3) for 10 minutes in 96 well plates. The ATP content in the cells was monitored at various concentration and time intervals by luciferase assay in a plate reader (Tecan, GENios) using manufacturer protocols. Cells were also allowed to recover ATP by the addition of growth media in the wells for 10 minutes before determining the ATP content. Based on these results, the optimum concentration for ATP depletion was determined to be 10 mM 2DG and 10 mM NaN_3.

To determine the effect of ATP depletion on cytosolic and glycosomal pH regulation under acidic pH_e, PF T. *brucei* were first loaded with BCECF and F-PTS1 respectively. Cells were then depleted of ATP in CB pH 7.4 as described above and placed into a microscopic perfusion chamber. The buffer was then exchanged with CB pH 6.0 while imaged at 495/440 nm over time intervals for 40 min. Quantification was achieved by internal calibration. For studying the effects of ATP depletion on the glycosomal acidification response, PF *T. brucei* were first incubated with F-PTS1 in mPBS for glycosomal pH quantification. The cells were placed in a perfusion chamber and buffer exchanged with nutrient-free mPBS containing 2DG and NaN_3 while imaging at 495/440 nm at intervals over 60 min. Final pH quantification was determined by internal calibration.

Inhibitor effect on pH regulation. PF *T. brucei* cells were pelleted and resuspended in nutrient-free mPBS containing F-PTS1 using standard protocols as described previously. The cells were then placed into a microscope perfusion chamber. Additionally, inhibitors (bafilomycin, 2 µM; vanadate, 500 µM; EIPA, 100 µM) were introduced into the incubation buffers individually or in combination. The cells were monitored for glycosomal pH by 495/440 nm emission of the localized F-PTS1 at intervals for 30 minutes by which all cell glycosomes exhibit a steady state pH. Results were compared to cells incubated in nutrient-supplemented mPBS under similar conditions as control.

Conflict of interest: The authors declare that they have no conflicts of interest with the contents of this article.

Author contributions: SL designed, performed and analyzed experiments shown in the manuscript. All authors aided in the interpretation of results and aided in drafting and editing the manuscript. All authors reviewed the results and approved the final version of the manuscript.

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FOOTNOTES

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[1]To whom correspondence should be addressed: Kenneth A. Christensen, Department of Chemistry and Biochemistry, Brigham Young University, C-205 BNSN, Provo, UT 84602. E-mail: kenc@chem.byu.edu; Tel: 801-422-0249.
[2]The abbreviations used are: PF, procyclic form; V-ATPase, vacuolar H^+ ATPases; P-ATPase, P-type ATPase; NHE, Na^+/H^- exchangers; F-PTS, fluorescein peroxisomal targeting sequence; pH_e, external pH (pH of incubation buffer); EIPA, 5-(N-ethyl)-N-isopropylamiloride; DMSO, dimethyl sulfoxide; BCECF, 2',7'-Bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein; 2DG, 2-deoxy-D-glucose; mPBS, Voorheis's modified PBS; CB, calibration buffer; PIP2, plasmalemmal phosphatidylinositol 4,5-bisphosphate; TbHK, *T. brucei* hexokinase.

FIGURE LEGENDS

Figure 1. Glucose and proline starvation results in glycosomal acidification. PF T. brucei cells were incubated (27 ºC, 5% CO2,) with F-PTS1 for 2 hours in mPBS with (A) and without (B) the respective nutrients before analysis. (A) The change in T. brucei glycosomal pH after buffer exchange to nutrient-free mPBS from mPBS containing 10 mM glucose, proline, or both. (B) The change in T. brucei glycosomal pH after the addition of mPBS containing 10 mM glucose, proline, or both to cells pre-incubated in starvation conditions for 2 hours. All pH values were calculated with from 495/440 nm emission ratio of F-PTS1 with an internal calibration. Error bars in both figures represent SEM from 25-50 cells.

Figure 2. Glycosomal pH is regulated independently from cytosolic pH and is more resistant to external acidic pH. (A) The glycosomal and cytosolic pH after incubation in nutrient-supplemented CB at pH_e 5.0, 6.0, and 7.4 for 1 hour. (B) Time chase analysis of this acidification process over 60 min. Vertical dotted line represents point of buffer exchange from pH_e 7.4 to pH_e 6.0 and 5.0. (C) Glycosomal and cytosolic pH in nutrient-supplemented CB pH_e 5.0 under digitonin titration. All pH values were calculated with from 495/440 nm emission ratio of F-PTS1 for glycosomes and BCECF for cytosol with an internal calibration. Error bars in both figures represent SEM from 25-50 cells.

Figure 3. Cytosolic and glycosomal pH regulation is compromised under K^+ or Na^+ - free buffers, respectively. (A) Cytosolic and (B) glycosomal pH after 1 hour incubation in Na^+ or K^+-free nutrient-supplemented CB at pH 7.4, 6.0, and 5.0. Error bars in both figures represent SEM from 25-50 cells. (C) Glycosomal pH of cells incubated in mPBS + glucose and nutrient-free mPBS modified to only contain Na^+ or K^+ ions at pH 7.4 for 1 hour. The box plot represents 10-90th percentile, with outliers shown as points. The differences in glycosomal pH under Na^+ and K^+ buffers are statistically different by ANOVA analysis ($p<0.05$). All pH values were calculated with from 495/440 nm emission ratio of F-PTS1 for glycosomes and BCECF for cytosol with an internal calibration.

Figure 4. Treatment of *T. brucei* with 2-deoxyglucose and sodium azide rapidly depletes basal ATP beyond normal starvation conditions. (A) ATP depletion (black bars) of cells incubated in glucose-free PBS containing 0 – 50 mM 2DG and NaN_3 after 10 min incubation and ATP recovery (white bars) of the same cells after buffer exchange into growth media for 10 minutes. Horizontal dotted line represents ATP levels of untreated cells in growth media. (B) Time chase analysis of this ATP depletion and recovery process at intervals over the first 12 minutes. (C) Comparison of ATP levels in T. *brucei* incubated in media, nutrient-free PBS, nutrient-supplemented PBS, or nutrient-free PBS with 2DG and NaN_3 for 10 minutes. The ATP levels after recovery in cells incubated under nutrient-free PBS with and without 2DG and NaN_3 are also reported (gray bars). All ATP values were determined by a luciferase assay. Error bars in all figures represent SEM from treatments performed in triplicate.

Figure 5. ATP-depleted *T. brucei* have compromised resistance to acidic pH_e and equilibrate with cytosolic pH. (A) Time-chase analysis of glycosomal pH over 60 min in cells exposed to pH_e 6.0 under nutrient-supplemented CB and in nutrient-free CB containing 2DG and NaN_3 for ATP depletion. Dotted vertical line represents the time point of buffer exchange to pH_e 6.0. (B) Comparison of cytosolic and glycosomal pH in pH_e 7.4 and 6.0 CB under nutrient-supplemented and ATP depletion conditions. All pH values were calculated with from 495/440 nm emission ratio of F-PTS1 for glycosomes and BCECF for cytosol with an internal calibration. Error bars in both figures represent the SEM from 25-50 cells.

Figure 6. ATP depletion prevents glycosomal acidification in response to glucose starvation. Cells were incubated in mPBS containing 10 mM glucose while monitoring glycosomal pH. The buffer was then exchanged (noted by the dotted vertical line) into glucose-free mPBS with and without ATP depletion conditions. A time chase analysis of glycosomal pH was then monitored over 60 minutes using F-PTS1 495/440 nm emission ratio with an internal calibration. Error bars represent the SEM from 25-50 cells.

Figure 7. The inhibitors EIPA and bafilomycin together function to prevent glycosome acidification response to nutrient deprivation. (A) The effect of inhibitors on glycosomal pH acidification under starvation conditions. PF T. *brucei* cells were incubated in nutrient supplemented mPBS or nutrient free mPBS with various combinations of inhibitors for V-atpase (bafilomycin), P-atpase (vanadate) and NHE (EIPA) for 30 minutes. The asterisks note results that are significantly different by ANOVA analysis ($p<0.001$) from nutrient-free mPBS without inhibitors (second bar from left). (B) Time-chase analysis of glycosomal pH after the addition of EIPA and bafilomycin (at the point indicated by the vertical dotted line). The horizontal dotted lines represents the physiological pH of T. *brucei* grown in growth media. All incubations include 90 µM F-PTS1 to visualize and quantify glycosomal pH using 495 / 440 nm emission and internal calibration. Error bars on both figures represent SEM from at least 25 cells.

Figure 1

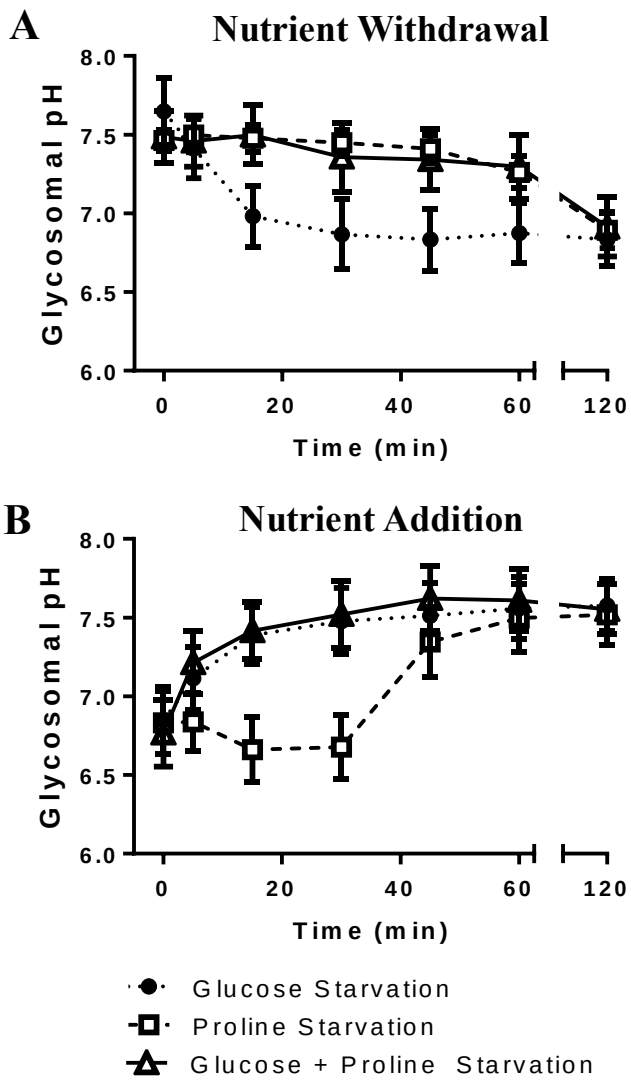


Figure 2

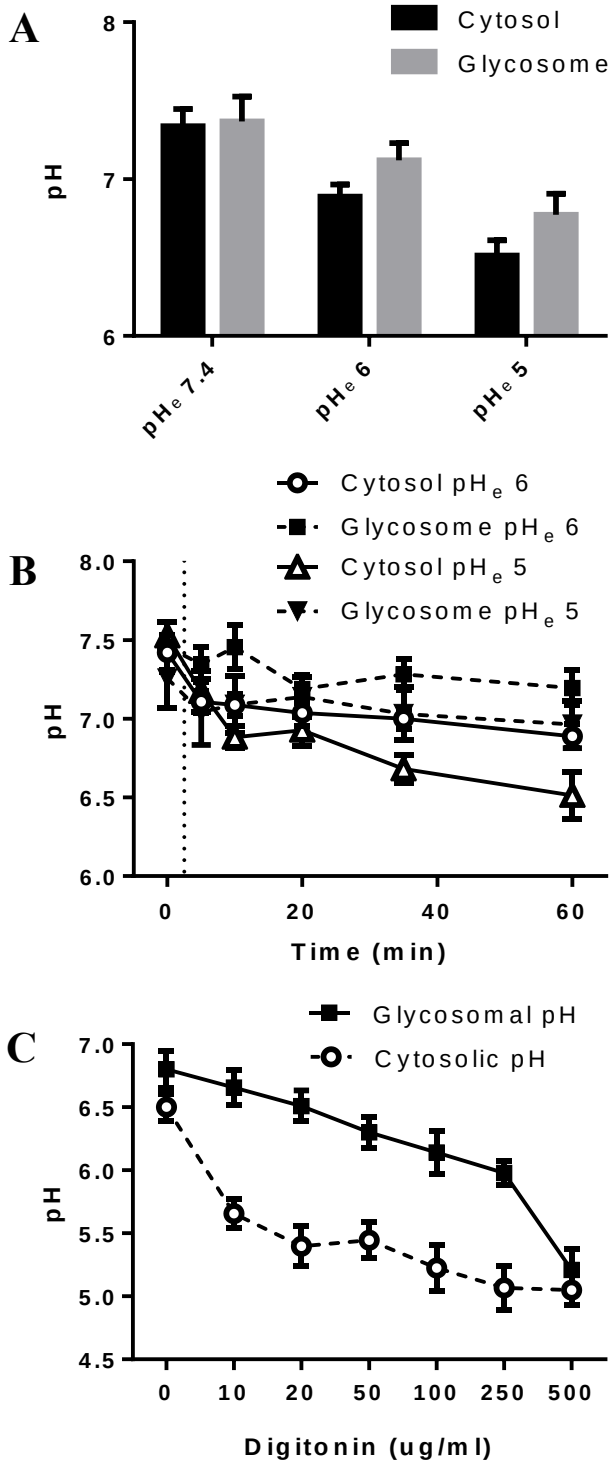


Figure 3

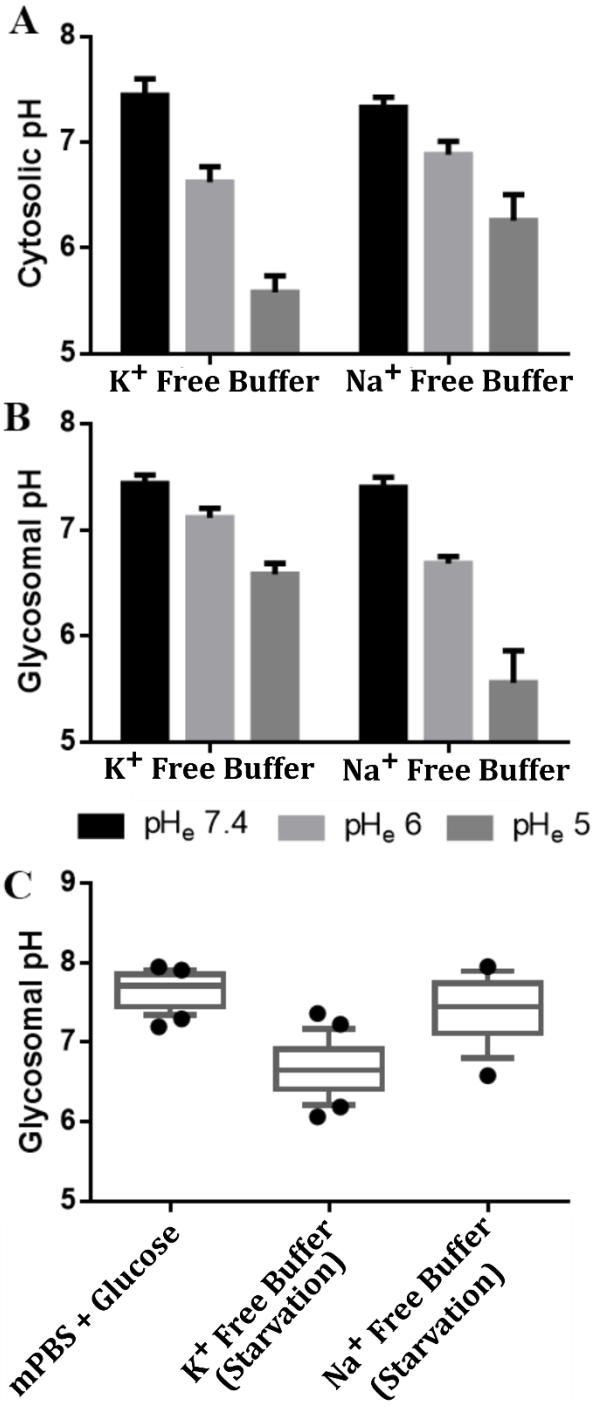


Figure 4

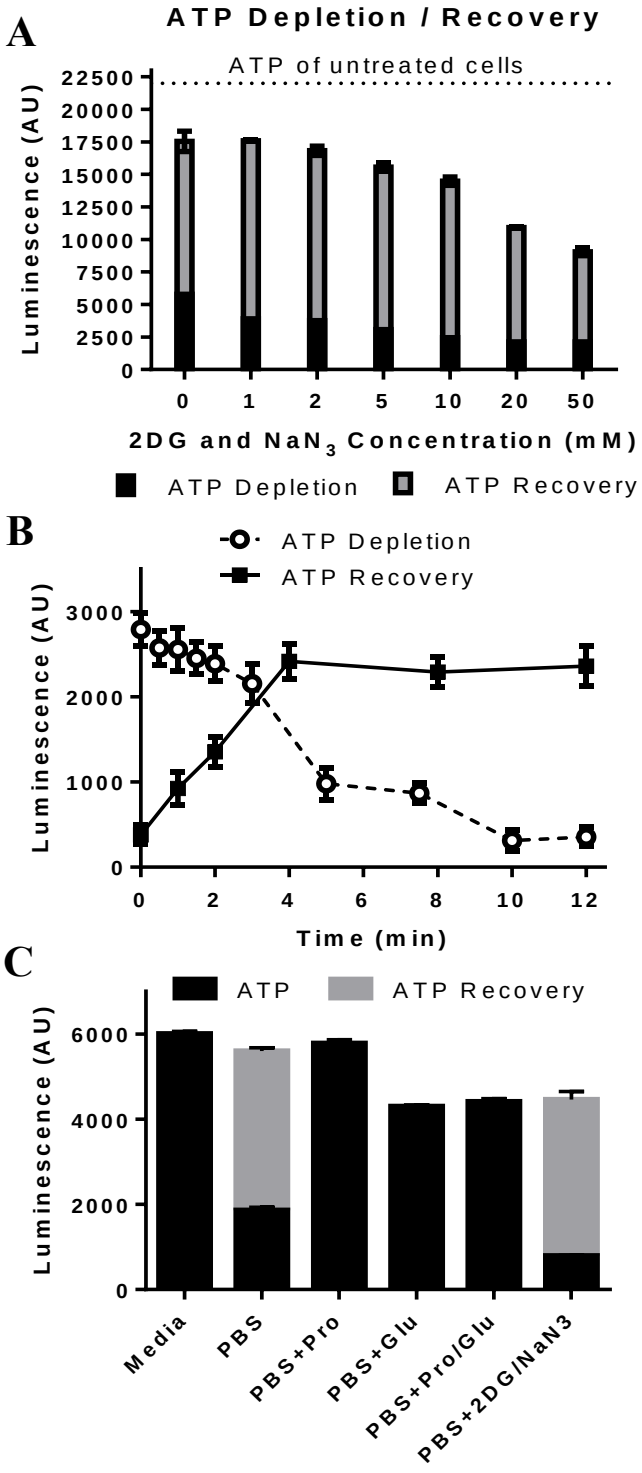


Figure 5

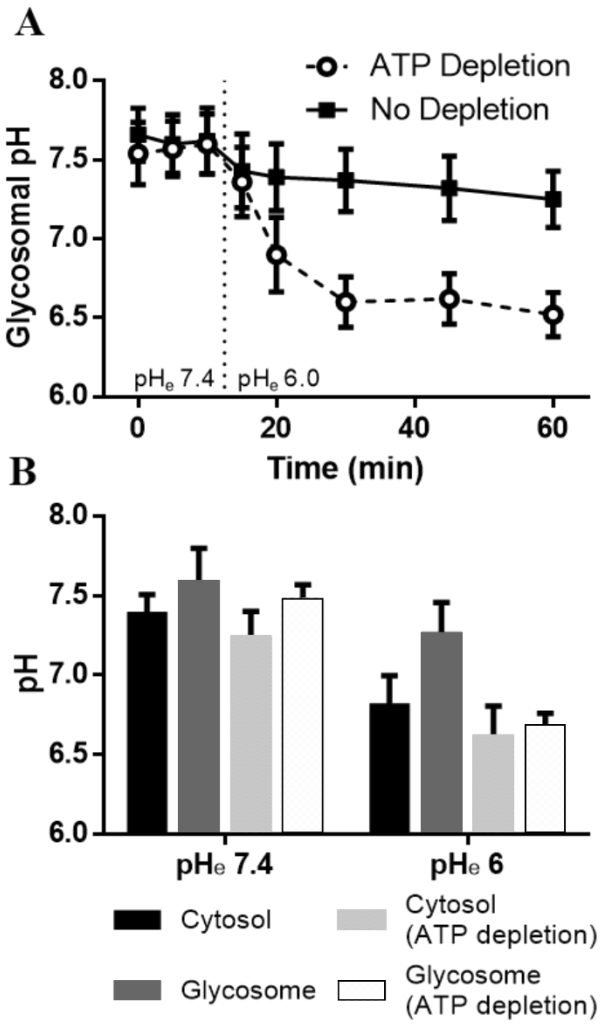


Figure 6

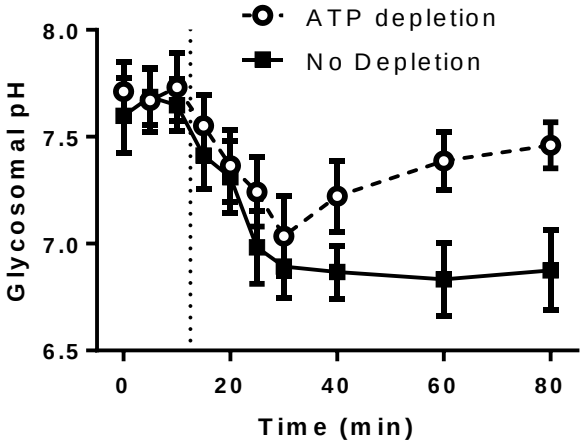
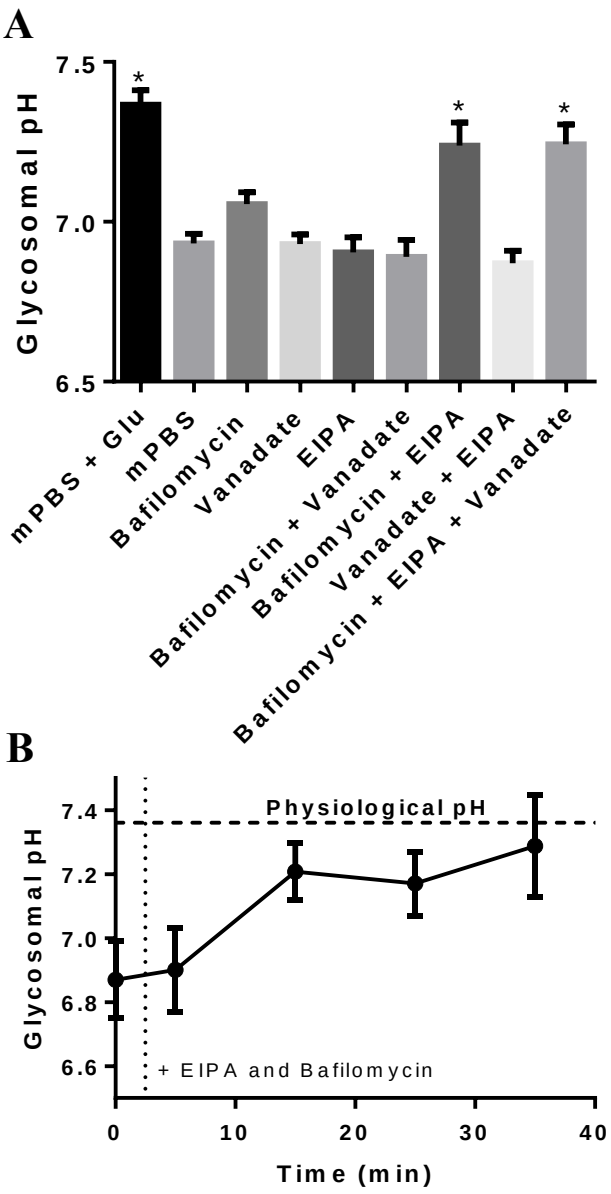


Figure 7



pH Regulation in Glycosomes of Procyclic Form *Trypanosoma brucei*

Sheng Lin, Charles Voyton, Meredith T. Morris, P. Christine Ackroyd, James C. Morris and Kenneth A. Christensen

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