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A determination of the steady state lysosomal pH of bloodstream stage African trypanosomes

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Abstract

The lysosomal/endosomal system of African trypanosomes is developmentally regulated and is important in the pathogenesis associated with infection of the mammalian bloodstream. Long considered to be a target for drug development, the internal pH of the lysosome has been variously reported to range from <5.0 to >6.0. We have refined a flow cytometric technique using a pH-sensitive probe that specifically targets the lysosome, tomato lectin:Oregon Green 488 conjugate. The probe is delivered to the lysosome with fidelity, where it is shielded against external pH. Measurement of fluorescent output in the presence and absence of lysotropic agent (NH₄Cl) then allows precise titration of steady state lysosomal pH (4.84 ± 0.23). Using bafilomycin A1 to inhibit acidification we demonstrate that this method is responsive to pharmacological perturbation of lysosomal physiology. This work should facilitate future studies of the lysosomal function in African trypanosomiasis, as well as other parasitic protozoa.

Keywords

trypanosome; lysosome; flow cytometry; pH

The eukaryotic lysosome has been traditionally defined as a discrete terminal degradative organelle of the endocytic pathway with a high density ($\rho = 1.10$ g/ml) and a low internal pH (≤ 5.0), [1]. It contains a unique complement of structural membrane glycoprotein markers (lysosome-associated membrane proteins; LAMPs), acid hydrolases (lipases, proteases, phosphatases and glycosidases), and a membrane proton pump (V-ATPase) that is responsible for luminal acidification. In African trypanosomes the lysosome is a single discrete organelle located at a perinuclear position in the posterior end of the cell. Morphologically it is indistinguishable between the procyclic insect and mammalian bloodstream stages. It too is defined by a LAMP-like membrane glycoprotein called p67 [2,3], acidic hydrolases such as cathepsin-L and -B orthologues [4–7], and a recent survey of the genome data base revealed a full complement of V-ATPase subunit orthologues [8]. It is well established that endocytosis is greatly upregulated in the bloodstream stage relative to procyclic forms [9–11], and this is mirrored by upregulation of lysosomal hydrolytic activities [6,7,9,12]. This difference at least in part reflects issues of nutrient acquisition for each life cycle stage. Procyclic cells can rely on the hydrolytic environment of the tsetse midgut to provide solutes for transport, while the bloodstream stage must aggressively take up and digest host serum macromolecules. A secondary consideration is immune evasion; potentially lytic or opsonic immune complexes

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are rapidly eliminated from the cell surface and degraded in the lysosome [13–15]. Whatever the true reason, lysosomal function is clearly critical for the success of the pathogenic bloodstream stage, and chemotherapeutic targeting of lysosomal protease activities is a real possibility [16]. In addition, the completion of the trypanosome genome will facilitate the identification of new components of the parasite lysosome and it will be desirable to modulate the expression of these proteins by gene knockout and RNAi strategies [8].

Toward these ends a better understanding of lysosomal physiology in bloodstream trypanosomes is essential, and one area where there has been longstanding uncertainty is the most fundamental property of all - internal pH. Three estimates have been published ranging from <5.0 [17], to ~5.4 [18], to 6.2 [19]. The two former methods were based on accumulation of weak amines that cannot discriminate between the lysosome and other acidic compartments such as endosomes and the acidocalcisome. The later method relied on uptake and lysosomal targeting of pH-sensitive fluorescein-lectin conjugates, and for reasons discussed below likely resulted in an artificially high pH estimate. Nevertheless we have adopted the same general methodology here since it has the key feature of specifically targeting the pH-sensitive probe to the lysosome.

Tomato lectin (Vector Laboratories, Burlingame CA) was conjugated with Oregon Green 488 carboxylic acid (5-isomer) succinimidyl ester according to the manufacturer's instructions (OG488, Molecular Probes, Eugene, OR). Fluorescent output of OG488 ($pK_a \sim 4.7$) is directly proportional to pH over a range typically found in eukaryotic lysosomes. Cultured 427 Lister Strain bloodstream trypanosomes were incubated with the tomato lectin:Oregon Green 488 conjugate (TL:OG488; 5 $\mu\text{g}/\text{ml}$) in serum-free HMI9 media supplemented with 0.5 mg/ml BSA at 37°C for 30 minutes. After washing, cells were incubated for an additional 5 minutes to chase the label into the lysosome. Inspection by epifluorescence microscopy confirmed that the probe predominantly colocalized with the lysosomal marker p67 (Fig 1A). Cells were washed into physiological MES-buffered saline of variable pH (50 mM MES, 50 mM NaCl, 5 mM KCl, 70 mM glucose, pH 4.25–6.75) containing DAPI (5 $\mu\text{g}/\text{ml}$). All samples were held on ice until flow cytometric analysis at ambient room temperature. Mean green fluorescent intensities (λ_{ex} 488 nm/ λ_{em} 524 nm) were determined by gating on viable DAPI-negative cells. Ammonium chloride (5 mM final) was then added to neutralize internal acidic compartments and mean fluorescent intensities of DAPI-negative cells were again determined. Sample handling was designed to normalize/minimize the length of time of exposure of live cells to low pH and lysomotropic agent. In addition, control experiments confirmed that no ambient photobleaching occurred during the course of acquiring a full flow cytometric data set, and that the ammonium chloride concentration was sufficient for rapid and maximal neutralization of lysosomal pH (data not shown). Typical scattergrams of DAPI vs OG488 fluorescence for high, middle and low external pH samples, with and without ammonium chloride are presented in Figure 1B. Viable DAPI-negative/OG488-positive cells lie in the lower right quadrant. Viability was excellent throughout (~95%) with only a modest drop off at the lowest external pH (pH 4.25), indicating that short-term exposure to low pH and/or lysomotropic agent did not have significant deleterious effects during the experimental manipulations. 10^4 DAPI-negative/OG488-positive events were collected for each mean fluorescent intensity (MFI) determination, and raw values were corrected by subtraction of green channel autofluorescence signals from unlabeled cells. Autofluorescence typically ranged from <10% to ~3% respectively for lowest and highest external pH.

Corrected OG488 MFIs were plotted vs external buffer pH ($\text{pH}_{(\text{ext})}$) and representative results are presented in Figure 1C. A typical linear curve of shallow slope was obtained for the control untreated cells. Theoretically the slope of this line should be zero if the probe is quantitatively internalized and shielded from the external buffer. In practice this is difficult to achieve and a low level of surface bound probe that is not evident by microscopy usually remains. Although

the internal pool of fluor is in excess, the residual external pool is preferentially influenced by $\text{pH}_{(\text{ext})}$ resulting in a shallow slope. In our hands this effect was much more prominent when wheat germ agglutinin (WGA) or concanavalin A were used as the carrier lectin (data not shown). In all cases however, with the addition of the lysomotropic agent ammonium chloride the fluorescence of the internal pool of probe also became sensitive to the $\text{pH}_{(\text{ext})}$ and dominated the total signal. Consequently at high $\text{pH}_{(\text{ext})}$ total signal sharply increased and at low $\text{pH}_{(\text{ext})}$ the total signal decreased resulting in a new curve with a steeper slope. The intersection of the new line with the control line represents the point where $\text{pH}_{(\text{ext})}$ precisely matches the normal internal lysosomal pH [$\text{pH}_{(\text{int})}$]. Solving for the value of x when the MFIs of control and ammonium chloride treated cells are equal provides this value. We have performed seven independent replicates of this experiment and find that the steady state lysosomal $\text{pH}_{(\text{int})}$ of bloodstream trypanosomes is 4.84 ± 0.23 (mean $\pm$ s.d.). To confirm that this methodology is responsive to perturbations of internal lysosomal pH we pretreated bloodstream trypanosomes with bafilomycin A1, a specific V-ATPase inhibitor that blocks proton translocation [20]. This treatment had little effect on the slope of the control cell curve (Fig. 1D), but the titrated equivalence point in the presence of ammonium chloride was elevated to give internal pHs of 6.63 and 6.56 in two independent replicates of the experiment.

Our value for the steady state lysosomal pH of bloodstream trypanosomes is most closely in line with the estimate of Coppens et al. (1993), and is greater than one pH unit lower than that of Brickman et. al (1995) ($\text{pH } 6.2 \pm 0.1$) despite the use of similar methodology. Two major factors likely contribute to this discrepancy. First, Brickman *et. al.* used wheat germ agglutinin (WGA):fluorescein conjugate as the pH sensitive probe. We have used tomato lectin in this study, which in our experience is superior to WGA because it is both more efficiently taken up and more quantitatively delivered to the lysosome (data not shown). This better insulates the probe from external $\text{pH}_{(\text{ext})}$ in control cells (shallower slope) and insures that the final pH measurement is reflective of the lysosome alone rather than an average of the lysosome and endosomal compartments (lower equivalence point). More importantly, Brickman *et. al.* did not titrate the equivalence point of $\text{pH}_{(\text{int})}$ vs. $\text{pH}_{(\text{ext})}$ as we have done. Instead a single fluorescent output measurement of endocytically labeled cells buffered at physiological $\text{pH}_{(\text{ext})}$ (~ 7.4) was compared to an independent $\text{pH}_{(\text{ext})}$ standard curve prepared with surface labeled cells. However as discussed above, because WGA is not as effectively internalized there is a significant residual external pool of probe, and consequently the fluorescent output of labeled cells in our hands is sharply dependent on $\text{pH}_{(\text{ext})}$ resulting in a much steeper control cell slope. Thus, by extrapolating a single experimental measurement at physiological $\text{pH}_{(\text{ext})}$ to the standard curve an artificially high lysosomal pH is obtained. A final contributing factor was the use of fluorescein as the pH-sensitive probe, which has a pK_a (~ 6.4) that is well above the pH range found in most eukaryotic lysosomes [note that the current spectrum of pH-sensitive fluors were not available at that time].

In conclusion, we believe this methodology provides a confident determination of the steady state internal lysosomal pH of bloodstream stage trypanosomes. One advantage of this approach is that it does not require complicated laser and/or filter combinations that are needed for dual wavelength ratiometric techniques. In addition, titration of the equivalence point between $\text{pH}_{(\text{int})}$ and $\text{pH}_{(\text{ext})}$ provides internal pH calibration curves that allow direct comparison of different cell populations. This feature is particularly relevant to experimental situations where genetic manipulations might be expected to directly or indirectly influence lysosomal physiology. Finally it is worth noting that this methodology should be widely applicable to other parasite systems where lysosomal activity plays a role in pathogenicity.

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Figure 1.

Panel A. Subcellular localization of endocytosed TL:OG488. Bloodstream trypanosomes were loaded with conjugate as described in the text. Cells were fixed, stained with anti-p67 and appropriate secondary reagent, imaged and deconvolved as described in [3]. Merged DAPI/DIC, green channel (TL:OG488), red channel (p67), and three channel merged fluorescence images are presented. Yellow staining in the 3x merged image indicates colocalization of probe with lysosomal p67 marker (arrowhead). Panel B. Flow cytometry scatter plots of DAPI vs OG488 fluorescence are presented for three representative values across the full range of pH_(ext) titration, with (bottom) or without (top) 5 mM ammonium chloride. DAPI-negative, OG488 positive cells are in the lower right quadrant, and % viable (DAPI-negative) cells are

indicated. 10^4 events in this quadrant were scored for every mean fluorescent intensity measurement (MFI). Panel C. A representative $\text{pH}_{(\text{int})}$ determination is presented. MFI values are plotted as a function of $\text{pH}_{(\text{ext})}$ for control (λ) and ammonium chloride treated (ν) samples. Curve fitting (linear regression) and statistical analyses were performed with Prism 4 software (GraphPad Software, Inc., San Diego CA). The calculated $\text{pH}_{(\text{int})}$ value for this independent experiment is indicated. Panel D. As in Panel C except cells were treated with bafilomycin A1 (2 μM) for 30 minutes prior to, as well as during, the uptake procedure.