Induced Activation of the *Toxoplasma gondii* Nucleoside Triphosphate Hydrolase Leads to Depletion of Host Cell ATP Levels and Rapid Exit of Intracellular Parasites from Infected Cells*

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The nucleoside triphosphate hydrolase of *Toxoplasma gondii* is a potent apyrase. The protein is synthesized in large amounts and transported through the secretory pathway of the parasite and into the vacuolar space in an oxidized and thereby enzymatically inactive form. Complete activation of the purified enzyme is known to require dithiols (e.g. DTT); subcellular fractionation demonstrates that little if any (<5%) of the enzyme in the vacuolar space is active in the absence of DTT. Both native and epitope-tagged nucleoside triphosphate hydrolase (NTPase) were partially activated during immunoprecipitation, precluding precise assessment of enzyme activity in the vacuolar space but suggesting that protein-protein interactions may trigger activation. When infected cells were treated with DTT, the NTPase was activated in a dose-response fashion, as assessed by migration on SDS-polyacrylamide gel electrophoresis and by an increase in enzymatic activity. After activation, enzyme activity decreased with time in the presence of DTT; this inactivation was slowed by the presence of excess ATP. A rapid fall in host cell ATP was accompanied by an abrupt exit of parasites from cells. These results demonstrate that the oxidation/reduction status of the NTPase, the only parasite dense granule protein that contains disulfide bonds, is tightly controlled within the vacuolar space and may influence parasite exit from cells.

*Toxoplasma gondii* is an obligate intracellular parasite and a significant pathogen of immunocompromised patients, notably persons with AIDS (1). The parasite enters target cells by a process of active invasion and resides within a specialized parasitophorous vacuole membrane (2, 3) (PVM)\(^1\). The PVM contains a pore. These considerations suggest that the enzymatic activity of the NTPase may be more complex. The enzyme contains disulfide bonds, and it is suggested that the enzyme may function in the purine salvage pathway by participating in the generation of the preferred purine salvage substrate, adenosine (9), according to the following scheme. Host cell ATP entering the vacuolar space via the PVM pore is degraded by the NTPase to ADP and AMP, with subsequent conversion to adenosine via 5\(^{-}\) nucleotidase and entry into the parasite via the *Toxoplasma* low affinity adenosine transporter (2, 10). (Free adenosine is normally present in relatively low levels in the eukaryotic cytoplasm, below the \(K_m\) of the transporter).

Several factors, however, suggest that the physiological function of the NTPase may be more complex. The enzyme contains 15 cysteine residues, and in vitro, requires dithiols to be enzymatically active. Dithiothreitol (DTT) will activate the enzyme, whereas monothiols such as glutathione and cysteine are ineffective (5). Thioredoxin is reported to activate the protein (11); however, host cell thioredoxin is presumably excluded from the parasitophorous vacuole by the PVM, and it is not known whether *Toxoplasma* secretes a physiologic dithiol. It is therefore unclear whether the secreted NTPase is actually active in the vacuolar space. Indeed, theoretical considerations suggest that it cannot be fully active. The NTPase constitutes 1–2% of total parasite protein (5); based on in vitro activity levels, we have calculated that the fully activated enzyme secreted by a single parasite could degrade the entire host ATP pool in a matter of minutes, assuming free access to ATP via the PVM pore. These considerations suggest that the enzymatic activity of the NTPase must be tightly regulated and raise the question...
of why the parasite might produce so much excess potential activity.

We have undertaken a series of experiments designed to demonstrate the validity of our in vitro function and regulation of NTPase and to resolve apparent discrepancies between the two. Here, we demonstrate that the NTPase is oxidized within the T. gondii secretory pathway and that the vast majority remains oxidized after secretion into the presumably reducing environment of the vacuolar space. The addition of exogenous dithiols reduces and activates the remaining enzyme. Full-scale activation of the NTPase results in rapid depletion of host cell ATP levels and exit of parasites from the cell.

**EXPERIMENTAL PROCEDURES**

**Cell Lines and Parasites**—Vero cells were grown at 5% CO₂ in minimal essential medium supplemented with 7.5% fetal calf serum, 2 mM glutamine, nonessential amino acids, penicillin, and streptomycin (Vero medium). T. gondii RH strain tachyzoites were maintained by serial passage in the peritoneum of Swiss-Webster mice or in Vero cell monolayers as described previously (12).

Parasite transfection was performed as described (13). Preparation of stable lines, electroporated parasites were cultured in a Vero cell monolayer, and monolayers were washed three times at 4 °C with PBS, and cold PBS containing 20 mM aprotinin, 5 µM leupeptin, 1% Triton X-100, and 50 mM Tris-Cl, pH 8.3, 100 mM NaCl, 2 mM EDTA, 1% Triton X-100 plus 1 mM phenylmethylsulfonyl fluoride. After incubation at 4 °C for 1 h, tubes were placed on ice, and 1 ml of each reaction was loaded onto polyethyleneimine F-cellulose TLC plates (J. T. Baker, Phillipsburg, NJ) that had been prespotted with 1 µl of a mix containing 10 µM each ATP, ADP, and AMP. The plate was developed in 0.4 M LiCl, 1 mM formic acid. The ATP, ADP, and AMP spots were visualized under UV light and excised, and the associated radioactivity was determined by liquid scintillation in Ultima Gold scintillation fluid (Packard Instruments, Meriden CT).

For the time course assays described in Fig. 5, purified recombinant NTPase (1 µg) in HM (100 mM Hepes, pH 7.4, 30 mM (CH₃COO)₂Mg) was pretreated with DTT (concentrations and time varied; see legend to Fig. 5 for details) before the addition of ATP to 10 mM and 0.3 µCi of [³²P]ATP; alternatively, the preincubation buffer contained 10 mM ATP. After incubation for 10 min at 37 °C, the reactions were stopped by the addition of cold trichloroacetic acid to a final concentration of 1%; after incubation on ice for 15 min, samples were assayed by TLC as described above.

**Construction of NTP3-c-myc**—Two polymerase chain reaction fragments were generated. Fragment 1 was generated with the following primers: forward, GCGAGGGTTGTAATATGGG; reverse, CGGCAATCTCCGGAAATGAC. This fragment spanned the region from bp 1547 to bp 2030 in NTP3 (GenBank accession number U14324), included 15 bp of the c-myc sequence at the 3' end and contains a natural Nhel site near the 5' end. Fragment 2 was generated with the following primers: forward, GAGCAGAAGCTTATCTCGGAG; reverse, GCGCATATCTTCGACAG. This fragment was extended in the opposite direction from bp 2226 in the 3'-untranslated region to bp 2031 in NTP3 (containing the stop codon), included the 30-bp c-myc sequence at the 5' end, and contains a natural BssHII site near the 3' end. Fragment 1 was digested with Nhel and HindIII, the latter of which cuts within the introduced c-myc sequence. Fragment 2 was digested with BssHII and HindIII. These restriction fragments were then ligated in a triple ligation to NTP3 in pBluescript (pBSNPT3) (8) and digested with Nhel and BssHII. The construct was sequenced to confirm that the junction area was correct.

**NTPase Activity Assay on Immunoprecipitated Protein**—Vero cell monolayers were infected for 20–24 h with the RH or RH-NTP-c-myc strain of T. gondii (3 x 10⁴ parasites/dish). Parasites were labeled for 15 min with [³⁵S]methionine/cysteine free Dulbecco's minimal essential medium supplemented with 7.5% fetal bovine serum, 2 mM glutamine, Tris-Cl, pH 7.4, 100 mM NaCl, 2 mM EDTA, 1% Triton X-100, and 50 mM Tris-Cl, 30% ethanol, dried, and developed by autoradiography on Kodak X-Omat AR film. T. gondii NTPase Leads to Reduced ATP Levels

**REFERENCES**
before 25 µl of the aliquot were assayed for NTPase activity as described above. Alternatively, 50 µl of DTT-treated cell supernatant was clarified and sampled without the addition of detergent.

**Determination of ATP Levels**—Vero cell monolayers in 24-well plates were infected as described above. Monolayers were washed two times with PBS and once with HM. DTT was added in HM (times and concentrations varied; see figure legends for details). ATP was extracted by the addition of an equal volume of 2% trichloroacetic acid for 15 min at room temperature. Samples were neutralized by the addition of an equal volume of 0.1n Tris, pH 9.0, before ATP determination using a luciferin/luciferase system (Molecular Probes, Eugene, OR) according to the manufacturer’s instructions.

**DTT-induced Exit Assay**—Vero cell monolayers were seeded in 24-well plates on 12-mm glass coverslips at 1 × 10⁵/well 24 h before infection with 2 × 10⁵ strain Toxoplasma/well. Monolayers were treated with DTT as described for the ATP depletion assay. After incubation with DTT, however, the supernatant was removed, and the monolayer was fixed and permeabilized with cold 100% methanol for 15 min at room temperature with no further washing. Detection was with mouse monoclonal antibody T6—2H11 against GRA3 (1:200) (17) and rabbit polyclonal antisera against whole T. gondii (1:250) diluted in 3% bovine serum albumin in PBS with 1 mM CaCl₂ and 1 mM MgCl₂. Secondary antibodies were fluorescein isothiocyanate-conjugated goat anti-mouse IgG and rhodamine-conjugated goat anti-rabbit IgG diluted 1: 500 in 3% bovine serum albumin in PBS with 1 mM CaCl₂ and 1 mM MgCl₂ using earlier procedures (16). Host and parasite nuclei were visualized by staining with 4,6-diamidino-2-phenylindole. Coverslips were mounted with Mowiol (Calbiochem) and observed with a Nikon Microphot FXA epifluorescence microscope. For quantitative assays, fields were chosen at random under 4,6-diamidino-2-phenylindole illumination and counted for host cells before parasites were counted under fluorescence isothiocyanate and rhodamine illumination. The percentage of infected cells (number of vacuoles/number of cells) and the average parasites/vacuole (total parasites counted/total vacuoles) were calculated. Selected images were photographed using Kodak Elite II ASA 400 color slide film, digitized with Sprintscan 35, and processed using Adobe Photoshop.

**RESULTS**

**The Oxidation State of NTPase Is the Same in the Parasite and the Vacuolar Space**—It has previously been reported (5) that a minute fraction (0.08–0.4%) of NTPase purified from host-free parasites is active in the absence of added DTT (DTT-independent). We sought to determine what fraction of NTPase in the vacuolar space is DTT-independent. We first assessed the oxidation-reduction status of the NTPase during transit through the T. gondii secretory pathway by immunoprecipitation of NTPase from host-free parasites. During the initial 15 min of the chase period, a diffuse band was observed, likely reflecting the presence of folding intermediates within the endoplasmic reticulum with incorrect or incomplete disulfide bond formation; at longer times the protein migrates as a single major band (Fig. 1A). Examination of NTPase from the vacuolar space shows that it co-migrates with NTPase from extracellular parasites (Fig. 1B). This result suggests that although the reducing environment of the vacuolar space is likely to be the same as that of the host cell cytosol, the majority of the enzyme in the vacuolar space is still fully oxidized; this is consistent with the low percentage of total NTPase that is active (see below).

**NTPase in the Vacuolar Space Has Little, if Any, Activity**—The failure to detect significant reduced NTPase in the vacuolar space suggests that the secreted enzyme is largely inactive. To examine this directly, ATPase activity was measured in detergent lysates. As shown in Table I, no consistent results were obtained in DTT-independent trials. In infected cells compared with uninfected cells (as previously demonstrated, uninfected cells have no detectable DTT-dependent ATPas activity). The occasional observation of increased activity (e.g., Table I, Exp. 2) may indicate that a portion of the NTPase is activated in vivo; alternatively, T. gondii infection may result in increased ATPase activity in the infected cell, or the activity may be due to other parasite proteins. The subcellular localization of the additional DTT-independent ATPase activity could distinguish among these possibilities. However, ATPase assays on subcellular fractions produced inconclusive and inconsistent results, precluding such an assignment (data not shown).

**NTPase Immunoprecipitated from the Vacuolar Space of Infected Cells May Be Activated by Antibody**—Because subcellular localization was inconclusive, we developed an alternative method to determine what fraction of DTT-independent ATPase activity in infected cells was indeed due to the NTPase. ATPase from the vacuolar space was immunoprecipitated using rabbit polyclonal antisera directed against recombinant NTPase or a mouse polyclonal antisera against an NTPase-glutathione S-transferase fusion protein (8), and the enzymatic activity was quantitated before and after treatment with DTT. As shown in Table I, approximately 30% of the total NTPase immunoprecipitated from the vacuolar space with murine anti-NTPase antiserum was active in the absence of added DTT. Although this might suggest that a substantial fraction of the NTPase in the vacuolar space was active, we have previously found that at least 25% of the total NTPase immunoprecipitated from extracellular parasites with mouse antiserum was DTT-independent (8), despite the fact that less than 1% of total

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**Table I**

| Trial | Lysate volume µl | NTPase activity | Infected/Uninfected | Uninfected DTT | Infected DTT |
|-------|------------------|-----------------|---------------------|--------------|--------------|
| 1     | 1                | 13.8            | 13.8                | 6.8          | 18.1         | 92.3         |
| 2     | 2.5              | 25.6            | 13.0                | 15.8         | 20.3         |
| 3     | 2.5              | 6.2             | 9.3                 | 9.4          | 104.9        |

**FIG. 1**

**NTPase is primarily in the oxidized form in both extracellular parasites and infected cells.** A, NTPase from host-free parasites was pulse-labeled and immunoprecipitated as described under "Experimental Procedures." B, infected Vero cell monolayers were treated with NEM, then scraped, and host cells were lysed by passage through a 27-gauge needle. Intact parasites were separated from host cell and vacuolar material by low speed centrifugation. Detergent extracts prepared from extracellular parasites (Par) or from the low speed supernatant (Vac) were analyzed by SDS-PAGE and immunoblotting in the presence or absence of 20 mM DTT as described under "Experimental Procedures." The positions of oxidized (Ox) and reduced (Re) NTPase are indicated.

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**Secreted NTPase has minimal, if any, activity in the absence of dithiols**

Detergent lysates were prepared from uninfected and infected Vero cell monolayers in NTPase buffer plus 0.5% Lubrol and assayed for ATPase activity in the presence and absence of 1 mM DTT as described under "Experimental Procedures." Data are presented for three independent trials.
parasite NTPase prepared by detergent lysate is DTT-independent. That result was confirmed in these experiments.

The surprisingly high DTT-independent NTPase activity in the immunopurified material may have been due to selective interaction between the antibody and activated NTPase. We therefore immunopurified NTPase from the vacuolar space via a c-myc epitope fused in-frame to the C terminus of NTPase3. This construct was stably expressed in the parasite and found by immunofluorescence to co-localize with native NTPase (18). Surprisingly, 35–60% of the total ATPase activity immunopurified from the vacuolar space with anti-c-myc antibody was DTT-independent (Table II). These results are analogous to those reported above after immunopurification with mouse anti-NTPase antiserum and suggest that the process of immunopurification may activate the NTPase in the absence of exogenous dithiols (see “Discussion”). Therefore, it was not possible to determine by immunopurification what fraction of NTPase, if any, was enzymatically active within the vacuolar space.

The NTPase Is Activated By the Addition of Extracellular DTT—Our data strongly suggest that the vast majority of NTPase in the vacuolar space is neither reduced nor active. We sought to determine whether the intravacuolar enzyme can be reduced by the addition of exogenous dithiols. In the presence of 1 mM NEM, treatment of infected cells with concentrations of DTT of 5 mM or greater resulted in a series of less rapidly migrating bands corresponding to reduced forms of the enzyme (Fig. 2). Increasing concentrations of DTT, through 50 mM, produced increasing amounts of reduced enzyme, although the extent of reduction was not being rigorously quantified.

The NTPase Is Activated By the Addition of Extracellular DTT—To demonstrate that the reduction seen in Fig. 2 corresponded to activation of vacuolar enzyme, we measured NTPase activity recoverable from infected monolayers treated for 10 min with DTT. Initially, NTPase activity was measured after Triton X-100 solubilization of DTT-treated monolayers (the presence of 1% Triton did not inhibit the activity of purified recombinant NTPase).3 As shown in Fig. 3A, the addition of DTT to infected, but not uninfected, cells results in significant levels of ATP-degrading activity. Because subsequent work demonstrated the lysis of host cells after DTT treatment (see below), we measured the NTPase activity present in the supernatants of monolayers after DTT addition but in the absence of detergent treatment (No Triton). As shown in Fig. 3A, the level of ATP-degrading activity detected in supernatants was essentially equivalent to that in detergent lysates, consistent with release by host cell lysis of most of the activated NTPase. To ensure that the activity was truly DTT-dependent, we demonstrated that the membrane-permeant alkylating agent NEM could prevent activation, consistent with in vitro findings (Fig. 3B).

We next determined the dose response to DTT treatment for

| Table II |
| NTPase immunoprecipitated from the vacuolar space may be activated by antibody |

| Experiment | Antibody | NTPase activity | nmol of ATP hydrolyzed |
|------------|----------|-----------------|------------------------|
|            |          | −DTT | +DTT |
| 1          | Rabbit anti-NTPase | 13 | 46 |
|            | Mouse anti-NTPase    | 197 | 606 |
|            | Mouse anti-c-myc     | 55 | 152 |
|            | Normal mouse serum   | 17 | 14 |
| 2          | Rabbit anti-NTPase | 36 | 39 |
|            | Mouse anti-NTPase    | 206 | 587 |
|            | Mouse anti-c-myc     | 92 | 132 |
|            | Normal mouse serum   | 31 | 42 |

3 J. A. Silverman, H. Qi, A. Riehl, C. Beckers, V. Nakaar, and K. A. Joiner, unpublished observations.
**in situ** activation of the NTPase. Maximum levels of NTPase activity are detected at 1 mM DTT, with decreasing activity in the presence of increasing concentrations of the reducing agent (Fig. 4). Based on dilutions of the 1 mM supernatant, we estimated the 50% sample to be 20-fold less active (data not shown). We obtained an identical result from detergent lysates of host-free parasites and for purified recombinant NTPase3 (data not shown), suggesting that the reverse dose response is an intrinsic property of the enzyme. These results are not affected by the inclusion of protease inhibitors nor did immunoblotting indicate any reduction (within 2-fold) in protein levels (data not shown), suggesting that the loss of activity is not due to degradation of the enzyme. This result is in direct contrast to the findings of Asai *et al.* (5), who reported no significant change in NTPase activity between 1 and 50 mM DTT. We have determined that the discrepancy in these observations is the result of the longer time of exposure to DTT in our assays due to a 10-min preincubation before enzyme assay. By varying the length of the preincubation, we have demonstrated that the NTPase is subject to a time-dependent, concentration-dependent inactivation after exposure to increasing concentrations of DTT (Fig. 5): $t_{1/2}$ ranges from >60 min at 1 mM to <10 min at 50 mM. Interestingly, the rate of inactivation of NTPase by DTT was altered by the presence of excess ATP in the reaction buffer (Fig. 5). Inclusion of 1 mM ATP increased the $t_{1/2}$ to >60 min at 10 mM and ~20 min at 50 mM, suggesting that the presence of substrate can protect the labile elements of the enzyme.

**Addition of DTT Leads to Depletion of Host Cell ATP Levels**—The presence of a large (M, cutoff ≤1000 daltons), relatively nonselective pore in the PVM should render the environment of the vacuolar space equivalent to that of the host cytosol and provide the parasite with free access to host cytosolic ATP pools. Activation of intracellular NTPase by DTT should therefore have an impact on host ATP levels. As shown in Fig. 6A, the addition of DTT at concentrations of >1 mM resulted in rapid depletion of ATP in infected cells, whereas treatment with up to 50 mM DTT had no effect on ATP levels in uninfected cells (trypan blue exclusion by uninfected cells was also unaffected [data not shown]). The dose response for this event closely paralleled that for reduction of the enzyme (Fig. 2), although running counter to that for *in situ* NTPase activation (Figs. 4 and 5). We also examined the kinetics of this process (Fig. 6B). Significant ATP reduction occurred within 1–3 min of DTT addition, consistent with our earlier calculations, strongly supporting the idea that NTPase has full access to host cytosolic ATP pools. After the initial rapid decrease, ATP levels in the population remained fairly constant (time ≥5 min). The level of ATP remaining was too high to be accounted for simply by the number of uninfected cells in the population and suggested that in some infected cells, no ATP depletion was occurring (see "Discussion").

Activation of NTPase *in vitro* was accomplished only by dithiols. Monothiols such as 2-mercaptoethanol and glutathione are ineffective. To examine whether monothiols could activate the enzyme *in situ*, ATP levels were measured after treatment of infected cells with 2-mercaptoethanol or glutathione. As shown in Fig. 7, treatment with up to 50 mM 2-mercaptoethanol or glutathione had no effect on ATP levels in infected as compared with uninfected cells (although extended incubation with 50 mM 2-mercaptoethanol reduced ATP levels in both somewhat [data not shown]).

As shown in Fig. 1, NTPase within the *T. gondii* secretory pathway was largely in the oxidized form. We examined the consequences of *in situ* activation of NTPase in host-free parasites. Treatment with 10 mM DTT for up to 60 min had no effect on parasite ATP levels (data not shown), consistent with the idea that the fully folded enzyme is stored within secretory granules and has no access to parasite cytoplasmic ATP pools or, alternatively, that it cannot be activated within the dense granule.

**Addition of DTT Leads to Parasite Exit from Cells**—We explored the consequences for *T. gondii* of rapid depletion of host ATP. Strikingly, within 1 min of DTT addition, intracellular parasites became motile and exited the vacuole and the cells, resulting in host cell lysis (Fig. 8). Pretreatment with NEM prevented DTT-induced exit, consistent with earlier results for NTPase activation (data not shown). The kinetics of this process closely parallel those for ATP depletion. A rapid reduction was seen in the percentage of cells infected (Fig. 9) with little or no additional lysis occurring after 10 min of DTT treatment. The exit phenotype is highly heterogeneous. Lysed and unlysed vacuoles are clearly visible in neighboring cells after 5 min of

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**Fig. 4.** The DTT dose response for *in situ* activation of NTPase is inversely related to the dose response for ATP depletion. Uninfected or infected Vero cell monolayers were treated with the indicated concentrations of DTT for 10 min. Cell supernatants were assayed for NTPase activity as described. Data shown are representative of four independent assays; all samples were prepared in duplicate.

**Fig. 5.** DTT-treated NTPase is subject to time-dependent inactivation. Purified recombinant NTPase3 was preincubated with DTT (concentrations and time as indicated) in the absence or presence of 1 mM ATP before assay as described under "Experimental Procedures." Data presented are the average of two independent assays.
DTT treatment (Fig. 8, 5-min sample, lower right). As shown in Fig. 10, there is no significant difference in the population distribution of parasites/vacuole before and after DTT addition, with the possible exception of vacuoles containing 32 parasites, suggesting that exit does not correlate with vacuole size.

**DISCUSSION**

The Toxoplasma NTPase is one of the most potent ATP degrading enzymes known. We have demonstrated here that the enzyme is found in a largely inactive, oxidized form in the parasitophorous vacuole, yet can be reduced and activated by DTT under these conditions. DTT activation results in rapid depletion of host ATP pools, consistent with our predictions, and results in exit of the parasites from the host cell. This phenotype is essentially identical to the previously described effects on infected cells of the calcium ionophore A23187 (19, 20), which suggested that changes in host cytoplasmic calcium levels may serve as a signal to initiate parasite exit. Stommel et al. (21) recently reported that DTT treatment of infected cells triggers calcium fluxes and parasite exit. Our data would suggest that the DTT-induced calcium flux is triggered by collapse of host cell ATP pools; however, the exact temporal sequence of events has not been established.

The exit of parasites after DTT treatment occurs very rapidly. Lysed vacuoles can be observed with 30 s of DTT addition, before significant drops in ATP have occurred in the population of host cells. In contrast, some parasites failed to exit after up to 60 min of DTT exposure (Fig. 9). We were unable to measure ATP levels on an individual cell basis, but the most likely explanation for these observations is heterogeneity in the response to DTT. Failure to respond to DTT treatment does not correlate with vacuole size but may be a result of low NTPase expression levels. Considerable variation in staining intensity is observed when infected monolayers are analyzed by immunofluorescence microscopy using anti-NTPase antibodies. Other possible factors leading to heterogeneity of the DTT response may include the host cell cycle stage, which is reported to influence the ability of Toxoplasma to invade tissue culture monolayers. Explanation of this phenotype will require a more complete understanding of the physiological role of NTPase.

The vacuolar space surrounding intracellular T. gondii is presumed to be a reducing environment, equivalent to the host cell cytosol, due to the presence of a nonselective pore across the PVM. Unlike most eukaryotes, proteins secreted by the parasite will emerge from the oxidized parasite secretory pathway and enter the relatively reduced environment of the vacuolar space. This suggests that proteins secreted by T. gondii should either (a) be stably folded, such that a change in the oxidation-reduction status will not alter their conformation or (b) require reduction to fold into an active conformation within the vacuolar space. The former condition is most easily met if secreted proteins lack disulfide bonds; intriguingly, of the eight dense granule proteins sequenced to date, only NTP1 and NTP3 have more than one cysteine after cleavage of the signal sequence. These two proteins appear to meet the second con-
dition, i.e. their activity is strictly dependent upon reduction; however, the level of reduction achieved by the cytoplasmic environment does not appear to be sufficient to activate the enzyme. Our results suggest that it is unlikely that the NTPase is regulated solely by changes in the vacuolar reducing environment. We base this conclusion on two findings: 1) the failure of monothiols, including glutathione in vast excess of physiological levels, to activate the enzyme, and 2) the disastrous consequences to both host and parasite of unfettered enzyme activity. Instead, we suggest that the NTPase must be regulated in a more precise and pinpoint fashion, possibly as a result of protein-protein interaction. The large amounts of the enzyme secreted into the vacuolar space, coupled with the small fraction of enzymatically active protein, may indicate that the stoichiometry of interaction with another protein controls activity. The large amounts of the enzyme secreted into the vacuolar space, coupled with the small fraction of enzymatically active protein, may indicate that the stoichiometry of interaction with another protein controls activity. The most plausible explanation for these results is that antibody activates the enzyme during immunoprecipitation; alternatively, a small subset of activated enzyme is preferentially immunoprecipitated (inconsistent with the NTP-myc immunoprecipitation result) or an inhibitor may be removed by immunoprecipitation (inconsistent with the DTT dependence of purified recombinant NTPase). Immunoprecipitation of NTPase from metabolically labeled cells in the presence of cross-linking agents may help resolve this issue.

Although considerable attention has been paid to the question of how the NTPase is turned on in vivo, the data presented here may also be relevant to the question of how it is turned off. We have described a time-dependent inactivation of the enzyme, the rate of which appears to be influenced by the concentration of ATP. We have not determined if this process occurs in vivo; however, such a mechanism would provide an intrinsic protection against runaway enzyme activity. It might also explain why so much NTPase is produced, if the activity per molecule is limited. Self-inactivation of the enzyme might also be utilized during the conversion of intracellular parasites from the rapidly growing tachyzoite to the cyst-forming bradyzoite. This conversion event is accompanied by numerous changes in gene expression, including the down-regulation of

**Fig. 8.** DTT treatment induces parasite exit from infected cells. Infected cell monolayers were fixed, stained, and photographed as described either before (0') or after (1', 5') treatment with 10 mM DTT. Parasites are visible in the rhodamine channel in the fluorescence image, whereas the vacuolar membrane is visible in the fluorescein isothiocyanate channel.

**Fig. 9.** The kinetics of parasite exit closely follow the kinetics of ATP depletion. Infected Vero cell monolayers were treated with 10 mM DTT for the time indicated before being fixed, stained, and counted as described under "Experimental Procedures." The number of cells infected was determined and is expressed as a percentage of cells infected before addition of DTT (0 min). Data shown are representative of six independent trials.

**Fig. 10.** DTT-induced parasite exit does not correlate with vacuole size. Infected Vero cell monolayers were treated with 10 mM DTT for the time indicated, then fixed, stained, and counted as described under "Experimental Procedures." The distribution of vacuoles by size is shown. The average number of parasites/vacuole is shown in the box. Data shown are representative of five independent assays.
expression from the NTPase promoter (24). Self-inactivation might provide a means of ensuring that enzyme activity was similarly down-regulated.

The experiments described here were undertaken with the purpose of demonstrating that our in vitro understanding of the T. gondii NTPase is relevant to its in vivo function. In addition to having done so, we have also offered some insight into how the enzyme may be turned on and off in a controlled manner. Such pinpoint control, probably via protein-protein interactions, would allow the enzyme to function in purine salvage pathways or serve to couple the degradation of ATP to other processes within the vacuole (such as metabolite transport). However, we are still unclear as to the circumstances under which Toxoplasma might wish to unleash the full degradative power of its NTPase pools. One interesting possibility is that NTPase activation is related to parasite exit from cells, serving either as a trigger of host cell death or as a means of preventing ATP-mediated signaling after cell lysis (22). It should be noted, however, that this is unlikely to be a general mechanism for exit of apicomplexan parasites from cells, since T. gondii is the only apicomplexan that expresses a DTT-activated ATPase or apyrase (23).

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