An Inducible 3'-Nucleotidase/Nuclease from the Trypanosomatid Crithidia luciliae

PURIFICATION AND CHARACTERIZATION*

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Several species of protozoan parasites of the family Trypanosomatidae have a surface membrane-associated enzyme which is capable of hydrolyzing extracellular 3'-nucleotides and nucleic acids, thereby aiding in the acquisition of nutritionally required purines and P, from their hosts. In Crithidia luciliae, this 3'-nucleotidase/nuclease previously has been shown to be highly regulated as purine and/or P, starvation of this trypanosomatid leads to as much as a 1000-fold increase in enzyme activity. We have purified the enzyme to apparent homogeneity from detergent extracts of purine-starved C. luciliae by heparin-agarose chromatography followed by Mono Q and Mono S fast protein liquid chromatography. The enzyme had an apparent molecular weight of 43,000 and a pI of approximately 5.8. The enzyme displayed broad pH optima, with peaks at 8.0, for both nucleotidase and nuclease activities. The pH optima shifted to lower values when the activity was assayed in the presence of sulfhydryl reagents. The enzyme was most active with 3'-AMP and poly(A) in nucleotidase and nuclease assays, respectively. As a nuclease the enzyme hydrolyzed RNA at a faster rate than single-stranded DNA with no detectable hydrolysis of double-stranded DNA. The loss of enzyme activity which occurred upon storage at acid pH was prevented by the inclusion of Zn²⁺ in storage buffers. The physicochemical and kinetic properties of this trypanosomatid enzyme suggest that it is similar to the class I nucleases found in fungi and in germinating seedlings of higher plants.

An enzyme, originally identified as a 3'-nucleotidase, has been localized to the surface membrane of several members of the protozoan family Trypanosomatidae. These members include species of Leishmania (1-3) and African trypanosomes (4, 5) which are pathogenic to man, as well as the related Crithidia luciliae (6, 7), a nonpathogenic parasite of insects. Subsequent studies, based primarily on the ability to renature inactive, enzyme protein.

A remarkable feature of this enzyme in C. luciliae is that the level of enzyme activity increases up to 1000-fold when the organisms are maintained in the absence of purines and/or P, (6, 7). The increased activity correlates with the appearance of a 43-kDa surface-labeled protein which co-migrates with enzyme activity in one- and two-dimensional electrophoresis systems. The increase in enzyme activity is prevented by cycloheximide, an inhibitor of protein synthesis, and by actinomycin D, an inhibitor of RNA synthesis (7). However, it is not known if these metabolic inhibitors directly affect the synthesis of the enzyme protein itself or another protein which may modify the activity of a pre-existing, but inactive, enzyme protein.

This striking induction of activity in response to clearly defined, and easily manipulated, nutrient signals justifies the further study of this enzyme as a regulatory system in this family of protozoan parasites. As this induction is the only known example of such a regulated change in an enzyme or surface protein in these organisms, it may serve as a model for the differentiation events which accompany life cycle stages in these important parasites. To understand the molecular basis of the regulation at the genetic and biochemical levels it is essential to purify the enzyme to homogeneity. We report on the purification and also describe some of the physicochemical and enzymatic properties of the enzyme. The results obtained indicate that the enzyme has several properties in common with class I and other so-called single-strand specific nucleases from germinating plants (11-16) and fungi (17).

EXPERIMENTAL PROCEDURES

Materials—Tris base (ultrapure grade) was purchased from Schwarz/Mann; HEPES from Research Organics, Cleveland, OH; octyl-β-D-glucopyranoside from Behring Diagnostics; CHAPS from Polysciences, Inc., Warrington, PA; urea (ultrapure) from Bethesda Research Laboratories, Gaithersburg, MD; silver staining reagents (Rapid Ag kit) from ICN Biomedicals, Irvine, CA; acrylamide and bisacrylamide from Serva, Heidelberg, Federal Republic of Germany; SDS-PAGE buffers (BufferEze formula 2) from Kodak, Rochester, NY; Hanks' balanced salt solution without sodium bicarbonate from Gibco; bicinchoninic acid reagents from Pierce Chemical Co.; Q-

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1 The abbreviations used are: SDS-PAGE, sodium dodecyl sulfate-polycrylamide gel electrophoresis; HEPES, N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid; CHAPS, 3-[3-cholamidopropyl dimethylammonio]1-propanesulfonate; NEPHGE, nonequilibrium pH-gradient electrophoresis; FPLC, fast protein/peptide/polynucleotide liquid chromatography; MES, 2-(N-morpholino)ethanesulfonic acid; DTT, dithiothreitol.

2 Alleman, M. and Gottlieb, M. (1990) Exp. Parasitol., in press.
Sepharose Fast Flow resin, Mono Q HR 5/5, and Mono S HR 5/5 FPLC columns from Pharmacia LKB Biotechnology, Inc., Piscataway, NJ; mid-range prestained electrophoresis standards from Diversified Risten, Newton Centre, MA. All other reagents, including pH 3–10 carrier ampholytes and sodium salts of nucleic acid and nucleotide enzyme substrates, were purchased from Sigma. Single-stranded DNA was prepared by heating (100 °C, 5 min) double-stranded calf thymus Type I DNA.

Maintenance and Cultivation of Organisms—C. luciliae (American Type Culture Collection No. 90258) was maintained by weekly passage in RE III, a chemically defined medium (18) containing 0.1 mM adenosine as the sole purine source. Large-scale preparations of purine-starved organisms were prepared in a crude medium prepared without purines, centrifuged again, and resuspended in RE III without purines. Following incubation for 24–36 h in purine-free RE III, the cells were harvested at 4 °C by centrifugation and washed once in Hanks’ balanced salts solution. The washed cell pellets were frozen and stored at −80 °C prior to use.

All media were sterilized by autoclaving at 121 °C for 35 min. Adenosine was filter sterilized and added to sterile RE III for maintenance of cell cultures.

Determination of Protein Concentrations—Protein concentrations were estimated colorimetrically by using bicinchoninic acid (20), or by measuring the absorbance of column eluates at 280 nm.

Enzyme Assays Standard reaction mixtures for 3'-nucleotidase activity assays (100 µl) contained 100 mM HEPES, pH 8.5, 2.5 mM 3'-AMP, 0.1% CHAPSO, and an aliquot of enzyme. The 3'-nucleotidase reaction mixtures (100 µl) contained 100 mM Tris maleate, pH 6.0, 5 mM 3'-AMP, 0.1% CHAPSO, and an aliquot of enzyme. Reaction mixtures were incubated at 42 °C for 15 or 30 min, stopped by chilling in an ice-water bath, and the amount of P1 released was estimated as described by Lanzetta et al. (21). One unit of enzyme activity is defined as 1 µmol of P1 released from the nucleotide substrate by the enzyme/min. Nuclease activity was determined by measuring the absorbance at 260 nm of acid-soluble material released from poly(A) substrate by the samples as described by Gottlieb et al. (7). One unit of nuclease activity is defined as 1 µmol of acid-soluble material released from the nucleic acid substrate by the enzyme/min assuming a molar extinction coefficient of 15,400. Acid phosphatase activity was determined by the fluorometric assay described by Grew et al. (22) using 4-methylumbelliferyl phosphate as substrate. Enzyme samples were diluted before assays, when necessary, in 30 mM HEPES, pH 8.0, containing 0.1% CHAPSO.

Gel Electrophoresis and Staining—SDS-PAGE was conducted according to the method of Laemmli (23) using 3% acrylamide in the stacking gel and 10% acrylamide for the separating gel. Two-dimensional gels were run as described by O’Farrell et al. (24) using NEPHGE with pH 3–10 carrier ampholytes in the first dimension and 10% SDS-PAGE in the second dimension. Samples were dissolved with gentle heating without reducing agents before NEPHGE in order to maintain the potential for enzyme activity staining after electrophoresis. The pH positions of the first dimensional electrophoresis were estimated by measuring the pH of slices taken from duplicate blank NEPHGE tube gels electrophoresed under identical conditions. Gels were stained for 3'-nucleotidase activity or nuclease activity following one- or two-dimensional SDS-PAGE as described previously (7) or were stained for protein using the Rapid-Ag silver staining kit (ICN) according to the manufacturer’s instructions. The protein molecular weight standards used were phosphorylase b (95,000), glutamate dehydrogenase (55,000), ovalbumin (45,000), lactate dehydrogenase (36,000), carbonic anhydrase (28,000), lactoglobulin (18,400), and cytochrome c (12,400).

Detergent Extraction—All purification procedures were carried out at 0 °C unless otherwise stated. The purification routinely was monitored by 3'-nucleotidase activity assay.

Frozen C. luciliae were lysed by resuspending, by vortexing at room temperature, cell pellets which contained a total of 3.7 × 10⁷ organisms in hypotonic lysis buffer (10 mM Tris HCl, pH 7.4, containing 2 mM phenylmethylsulfonyl fluoride, and 5 µg/ml leupeptin) to a density of 10⁷ cells/ml. The lysate was centrifuged at 10,000×g for 50 min. The resulting pellets were homogenized usually in a tissue grinder in 200 ml of 10 mM Tris-HCl, pH 7.4, containing 0.3 M NaCl, and centrifuged at 15,000×g for 30 min. The salt-washed pellets were homogenized in 125 ml of 20 mM HEPES, pH 6.0, containing 5 µg/ml leupeptin, and octyl-β-D-glucopyranoside. The homogenate was extracted for 1 h with rocking, centrifuged at 150,000 g for 75 min, and the supernatant fluid (octyl-β-D-glucopyranoside extract) was removed.

Heparin-Agarose Chromatography—The octyl-β-D-glucopyranoside extract (130 ml) was adjusted to pH 6.0 immediately before heparin-agarose chromatography by the addition, with stirring, of 0.5 M acetic acid. The enzyme did not adsorb significantly to the column matrix at pH values greater than 6.5. The extract was applied (flow rate 1.5 ml/min) to a 2.5 × 20-cm column of heparin-agarose equilibrated in 50 mM sodium acetate, pH 6.0, containing 0.5% Triton X-100 and 5 µg/ml leupeptin (Buffer A). The column was washed with 120 ml of Buffer A prior to elution with a 150-ml linear gradient of 0–0.7 M NaCl in Buffer A. Fractions (4 ml) were collected and assayed for 3'-nucleotidase activity and protein. Fractions which exhibited a specific activity greater than that of the starting material were combined and designated the heparin-agarose pool (59 ml).

Q-Sepharose Chromatography—A portion (48 ml) of the heparin-agarose pool was diluted 1:15 in 20 mM MES, pH 6.5, with 0.5% Triton X-100 (Buffer B) and applied (flow rate 1.5 ml/min) to a 2.5 × 6-cm Q-Sepharose column equilibrated in Buffer B. The column was washed with 120 ml of 20 mM MES, pH 6.5, containing 1% octyl-β-D-glucopyranoside and 5 µg/ml leupeptin (Buffer C) and the enzyme eluted with a 150-ml linear gradient of 0–0.35 M NaCl in Buffer C. Fractions (4 ml) were collected and assayed for 3'-nucleotidase activity. The capacity of the Q-Sepharose column made it suitable for large-scale purifications of the enzyme.

Mono S FPLC—Mono Q FPLC was employed as an alternative to Q-Sepharose chromatography in some experiments because the Mono Q system provided better resolution, albeit with smaller capacity, than Q-Sepharose. Five milliliters of the heparin-agarose pool was concentrated to 0.3 ml in a Centriprep 30 microconcentrator (Amicon), diluted to 16 ml in Buffer C, and applied (flow rate of 1 ml/min) to a 1-ml Mono S FPLC column. The column was washed with 25 ml of Buffer C and the enzyme eluted with a 40-ml linear gradient of 0–0.4 M NaCl in Buffer C.

Mono S FPLC—The Q-Sepharose pool (71 ml) was concentrated 35-fold in a Centriprep 30 microconcentrator, diluted 10-fold in 20 mM MES, pH 5.0, containing 1% octyl-β-D-glucopyranoside, 5 µg/ml leupeptin, and 0.3 mM ZnSO₄ (Buffer D) and applied (flow rate 1 ml/min) to a 1-ml Mono S FPLC column. The column was washed with 25 ml of Buffer D and eluted with a 40-ml linear gradient of 0–0.4 M NaCl in Buffer D. Mono Q FPLC-purified enzyme was chromatographed separately in an identical manner.

RESULTS

Purification of Crithidial 3'-Nucleotidase/Nuclease

The crithidial 3'-nucleotidase/nuclease was purified from organisms which were maintained for 24–36 h in purine-deficient medium. As previously reported, purine-starved crithidia exhibited 1000-fold more enzyme activity than purine-replete organisms (6, 7). The purification was monitored by 3'-nucleotidase assay rather than nuclease assay because of the greater sensitivity and convenience of the former. In addition, we analyzed samples from the purification by activity staining gels after SDS-PAGE. The enzyme, as demonstrated by either 3'-nucleotidase or nuclease activity staining, migrated with an apparent molecular weight of 43,000. When the purification was carried out in the absence of protease inhibitors, bands of enzyme activity which migrated faster than 43 kDa appeared following SDS-PAGE of samples of the detergent extract and subsequent stages of the purification (data not shown). We attributed these bands to proteolytic digestion of the intact enzyme.

Approximately 95% of the 3'-nucleotidase activity was associated with the membrane fraction (15,000 × g pellet) after centrifugation of whole cell lysates (Table I). This finding was consistent with the previous localization of the enzyme to the membrane fractions (1, 32, 33). The requirement for detergents such as octyl-β-D-glucopyranoside or Triton X-100 for solubilization of the enzyme from membrane pellets and for maintaining solubility of the enzyme during purification and enzyme assays suggested that the 3'-nucleotidase/
nuclease is an integral membrane protein.

**Heparin-Agarose Chromatography**—During chromatography of the detergent extract on heparin-agarose, the enzyme eluted over a broad range of the salt gradient with peak elution at 0.35 M NaCl. The specific activity of the pooled active fractions was 2-fold greater than that of the octyl-β-D-glucopyranoside extract. This step separated the enzyme from a periodic acid Schiff-staining substance which did not adsorb to the column (data not shown). This substance was presumably a lipopolysaccharide, analogous to a previously described arabinogalactan from *Crithidia fasciculata* (19) and to the lipophosphoglycan of *Leishmania* spp. (25, 26).

**Ion Exchange Chromatography**—A portion of the heparin-agarose purified material was chromatographed on Q-Sepharose as described under “Experimental Procedures.” Fractions 20–36 (denoted by the bracket) were combined to form the Q-Sepharose pool.

**Adsorption of the enzyme to the Q-Sepharose (anion exchange) column decreased at pH values lower than 6.5 and adsorption to the Mono S column (cation exchange) decreased at pH values greater than 5.0 (data not shown).** These observations were consistent with the determination by two-dimensional electrophoresis that the pI of the enzyme was approximately 5.8 (see below).

### Table I

| Step                        | Protein mg | 3'-Nucleotidase activity units | Specific activity units/mg | Purification* | Yield† | Nuclease activity units | Ratio‡ |
|-----------------------------|------------|-------------------------------|---------------------------|---------------|--------|------------------------|--------|
| Lysate                      | 1390       | 6540                          |                           | 4.7           | 1      | 100                    | 28     |
| 15,000 × g pellet           | 490        | 6220                          | 10                        | 2.7           | 95     | 274                    | 23     |
| Octyl-β-D-glucopyranoside extract | 153       | 4710                          | 31                        | 6.5           | 72     | 298                    | 16     |
| Heparin-agarose pool       | 61         | 3420                          | 56                        | 12            | 52     | 263                    | 13     |
| Q-Sepharose pool           | 14         | 2029                          | 144                       | 31            | 31     | 206                    | 9      |
| Mono S pool                | 2.0        | 1420                          | 710                       | 150           | 22     | 139                    | 10     |
| Mono Q-Mono S pool*        | 0.013      | 30                            | 2310                      | 490           | 5      | 6                      | 5      |

* Based on 3'-nucleotidase activity. Yields were corrected for aliquots removed during purification.  
† Ratio of 3'-nucleotidase specific activity/nuclease specific activity.  
‡ Sum of Mono S pools after chromatography of Mono Q pools 1 and 2.

In order to obtain better resolution than that which was possible on the large capacity Q-Sepharose column, a portion of the heparin-agarose purified material was subjected to Mono Q FPLC. The resulting column profile (Fig. 2) revealed two peaks of enzyme activity. These peaks were pooled separately, designated pool 1 and pool 2, and used for Mono S FPLC. Activity stained gel patterns revealed no differences between the enzyme collected in pool 1 and pool 2. The combined pools contained 77% of the 3'-nucleotidase activity loaded onto the column, and exhibited a 5-fold increase in specific activity (280 units/mg).

A column profile for the Mono S FPLC chromatography of the Mono Q FPLC pool 2 is shown in Fig. 3. Virtually identical results were obtained for pool 1 (data not shown). The active fractions from pools 1 and 2 were combined and are referred to as the Mono Q-Mono S pool. The gel pattern of the Mono Q-Mono S pool after SDS-PAGE revealed a single silver-stained band at 45 kDa which co-migrated with enzyme activity (Fig. 4).

**Purification Summary**—The yields and specific activities of the enzyme during each step of the purification are shown in Table I. Each step of the purification also was analyzed by SDS-PAGE followed by staining for protein and for 3'-nucleotidase activity (Fig. 4). Identical results were obtained when
The purified enzyme and crude octyl-$\beta$-D-glucopyranoside extracts of membranes were assayed for two other enzyme activities associated with trypanosomatid surface membranes, acid phosphatase, and 5'-nucleotidase (1, 3, 27). Neither of these activities were detected in Mono S-purified material, but both activities were present in the octyl-$\beta$-D-glucopyranoside extract (data not shown).

**Properties of the Purified Crithidial 3'-Nucleotidase/Nuclease**

**Two-dimensional Electrophoresis—**To confirm that the same enzyme was responsible for both 3'-nucleotidase and nuclease activities, two-dimensional electrophoresis of purified 3'-nucleotidase/nuclease (Mono S pool) was carried out. NEPHGE was used in the first dimension to resolve proteins over a broad pH range (pH 4.7–8.5). Following electrophoresis in the second dimension, the gels were silver-stained for protein or stained for nuclease or 3'-nucleotidase activity (Fig. 5). The gel staining patterns revealed co-migration of protein with nuclease and 3'-nucleotidase activities at a pI of 5.8 and an M, of 43,000. Similar gels, using isoelectric focusing (pH range 4.5–6.5) in the first dimension to obtain better resolution around pH 5.8, supported the conclusion that the same enzyme is responsible for both activities (data not shown).

**pH Optima—**The effect of pH on the 3'-nucleotidase and nuclease activities of the Mono S-purified crithidial 3'-nucleotidase/nuclease, when assayed under standard conditions, is shown in Fig. 6A. Similar results were obtained using a combination of 0.1 M NaAc (pH 4.5–6.5), MES (pH 5.0–7.0), and HEPES (pH 6.5–9.0) instead of Tris maleate to buffer the reactions. The results indicated broad pH optima, around pH 8.0, for both activities. When the crithidial enzyme was assayed in the presence of 1 mM DTT (Fig. 6B) to approximate the conditions under which plant class I nuclease activities were assayed in some reports (11, 13), a shift in the pH optima to lower values occurred. The shift in pH optima of the crithidial enzyme, from 8.0 in the absence of DTT, to 6.0–6.5 in the presence of DTT, may be explained by our finding that 1 mM DTT inhibited 85% of the enzyme activity when assayed at pH 8.0, but only 20% of the activity when assayed at pH 6.0 when compared to control assays without DTT (Fig. 7). Similar results were obtained using cysteine instead of DTT. The differential effects of thiol on the activity of the enzyme at pH 6.0 further indicate the involvement of cysteine residues in the enzyme's active site.
enzyme at various pH values will be considered in more detail below. The results were not influenced by the purity of the enzyme preparations (detergent extracts to homogeneous enzyme) used in repetitions of these experiments (data not shown).

**Substrate Specificity**—Table II shows the substrate specificities of the purified nuclease when assayed at pH 8.0 for 3'-nucleotidase and nuclease activities. The results revealed a preference for 3'-ribonucleotide substrates with no activity on either deoxyribonucleotides or cyclic nucleotides. The $K_M$ of the enzyme for 3'-AMP was approximately 0.4 mM; purified enzyme exhibited a $V_{max}$ of 1470 units/mg; and the maximum nuclease activity was 260 units/mg. The symbols used are: $\bullet$, 3'-nucleotidase activity; $\bigcirc$, nuclease activity.

**Table II**

| Substrate 3'-Nucleotidase activity $a$ | Substrate Nuclease activity $a$ |
|----------------------------------------|---------------------------------|
| % maximum                              | % maximum                       |
| 3'-AMP 100                             | Poly(A) 100                     |
| pAp 49                                 | Poly(U) 62                      |
| 3'-UMP 44                              | Poly(C) 5                       |
| 3'-GMP 6                               | Poly(G) 1                       |
| 3'-CMP 2                               | <0.1                             |
| 3'-TMP <0.1                            | RNA 55                           |
| 2'-AMP <0.1                            | Single-stranded DNA 6           |
| 5'-AMP <0.1                            | Double-stranded DNA <0.1        |
| 3'-dAMP <0.1                           |                                 |
| 3',5'-cAMP <0.1                        |                                 |
| 5'-ADP <0.1                            |                                 |
| 5'-ATP <0.1                            |                                 |
| Poly(A) <0.1                           |                                 |

$^a$ Purified crithidial 3'-nucleotidase/nuclease (Mono S pool) was assayed for 3'-nucleotidase activity at pH 8.0 as described under "Experimental Procedures." Reaction mixtures contained 5 mM of the indicated substrates (except poly(A), at 10 mg/ml) and approximately 20 ng of protein/ml.

**End Products of Digestion**—As indicated above, P, was not detected following the incubation of the purified enzyme with poly(A), this result was consistent with a predicted mechanism of hydrolysis which yielded nucleotides that possess terminal 5'-phosphate and 3'-hydroxyl groups as products. Further evidence that the products of poly(A)hydrolysis by the 3'-nucleotidase/nuclease were 5'-nucleotides was provided by the observation that the addition of purified snake venom 5'-nucleotidase, which specifically releases P, from 5'-nucleotides (28), resulted in the release of P, from the products of 3'-nucleotidase/nuclease digestion of poly(A) (data not shown).

Crude detergent extracts of crithidial membranes, in contrast to purified 3'-nucleotidase/nuclease, released significant amounts of P, from comparable levels of poly(A) when assayed in a similar manner. The results suggested that another enzyme(s) present in the octyl-{$\beta$}-D-glucopyranoside extracts, presumably including the aforementioned crithidial 5'-nucleotidase, was responsible for the released P,

**Inhibitor Sensitivity**—The crithidial 3'-nucleotidase/nuclease was not inhibited by commonly used phosphatase inhibitors including tartrate, molybdate, and fluoride ions when these inhibitors were included in standard reaction mixtures at a concentration of 1 mM. Under the conditions of such experiments, the activity was partially sensitive to 1 mM EDTA (40% inhibition), although incubation of the 3'-nucleotidase/nuclease with 1 mM EDTA prior to enzyme assays completely abolished enzyme activity. The activity loss caused by EDTA could be recovered by preincubating the enzyme before the assay for 30 min in 0.3 mM ZnSO$_4$, provided the ZnSO$_4$ was diluted to subinhibitory concentrations during the assay. The enzyme was extremely sensitive to $Zn^{2+}$ and sulfhydryl reagents in these assays at pH 8.0, as 1 mM ZnSO$_4$, 1 mM cysteine, or 1 mM DTT inhibited over 90% of the enzyme activity. The enzyme activity was inhibited to a much lesser extent when the assays were carried out at pH 6.0 as mentioned above for thiol reagents and in Fig. 7, and indeed $Zn^{2+}$ double-stranded DNA; however, the enzyme was capable of hydrolyzing single-stranded, denatured DNA, although to a lesser extent than RNA.

**Fig. 6**. pH optima for 3'-nucleotidase and nuclease activities of the crithidial 3'-nucleotidase/nuclease in the absence (A) and presence (B) of DTT. A, purified enzyme was assayed for 3'-nucleotidase and nuclease activities as described under "Experimental Procedures" except 0.1 mM Tris maleate at the indicated pH values was included as buffer in the reaction mixtures. The 3'-nucleotidase assays contained approximately 3.3 ng of protein/ml and the nuclease assays contained approximately 170 ng of protein/ml. The maximum 3'-nucleotidase activity was 1860 units/mg, and the maximum nuclease activity was 495 units/mg. B, assays were performed as described in A but included 1 mM DTT in the reaction mixtures. The maximum 3'-nucleotidase activity was 950 units/mg, and the maximum nuclease activity was 760 units/mg. PH indicated concentrations. Control values (445 units/mg at pH 6.0 and 760 units/mg at pH 8.0) were obtained by assay without DTT at the indicated pH.

**Fig. 7**. Differential sensitivity of the crithidial 3'-nucleotidase/nuclease to DTT at pH 6.0 and 8.0. Crithidial 3'-nucleotidase/nuclease was assayed for 3'-nucleotidase activity at pH 6.0 (C) or pH 8.0 (D) with DTT included in the reaction mixtures at the indicated concentrations. Control values (445 units/mg at pH 6.0 and 760 units/mg at pH 8.0) were obtained by assay without DTT at the indicated pH.
**Crithidial 3'-Nucleotidase/Nuclease**

We have purified a strongly regulated enzyme, originally described as a 3'-nucleotidase (6), from the trypanosomatid protozoan *C. luciliae*. We have shown that the enzyme also possesses nuclease activity based on the observations that the 3'-nucleotidase and nuclease activities co-purify and co-migrate in both one- and two-dimensional electrophoresis.

The properties of the enzyme described in this report are similar to those of a number of so-called single-strand specific nucleases (29), especially the class I nucleases, from germinating plant seedlings (15, 17). The similarity to these plant enzymes is based on a comparison of their enzymatic properties to those described by Wilson (16) in his classification of plant nucleases. These enzymes, like the crithidial 3'-nucleotidase/nuclease, are capable of hydrolyzing the 3'-phosphate group from nucleoside 3'-monophosphates, principally ribonucleotides, as well as hydrolyzing nucleic acids. The hydrolysis of RNA by this group of enzymes is greater than that of denatured DNA, which is greater than that of native DNA. The enzymes are inhibited by EDTA*, and have a requirement for divalent cations, frequently Ca**, for activity and/or stability. The plant nucleases I are generally recognized as having pH optima in the range of 5.0–6.8 (18, 17).

However, the plant nucleases usually have been assayed in the presence of varying, and frequently undisclosed, amounts of sulfhydryl reagents. Although the crithidial enzyme displays its greatest activity at pH 8.0, it does exhibit a lower pH optimum when assayed in the presence of thiols. The thiol-dependent shift in pH optima is due to the fact that thiols inhibit enzyme activity to a greater extent at alkaline pH than at acid pH in both the plant nucleases (30, 31) and in crithidia. Similar effects are observed with certain divalent cations. For example, Zn**, which is necessary for maintenance of enzyme activity during storage at acid pH, is inhibitory when present during enzyme assays at pH 8.0 and to a lesser extent at pH 6.0. In addition to enzymic properties, the enzymes from trypanosomatid protozoa and from plants are similar in size, with apparent molecular weights of approximately 40,000.

Despite the numerous similarities there are a number of properties of the protozoal enzyme that distinguish it from the analogous enzymes and make it an important subject for further investigation. The trypanosomatid 3'-nucleotidase/nuclease is the first such enzyme described from organisms incapable of synthesizing purines de novo. It is also the first enzyme of this group which has been definitively localized to the surface membrane of a cell (32, 33). Despite the suggestion that the plant enzymes may be particle- or membrane-associated (15), nucleases I are generally thought to be soluble and indeed some have been obtained from supernatant fluids of plant cell cultures (29).

**Discussion**

**Stabilization of Enzyme Activity by Zn**

We have purified a strongly regulated enzyme, originally described as a 3'-nucleotidase (6), from the trypanosomatid protozoan *C. luciliae*. We have shown that the enzyme also possesses nuclease activity based on the observations that the 3'-nucleotidase and nuclease activities co-purify and co-migrate in both one- and two-dimensional electrophoresis.

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**Based Upon Its Surface Membrane Localization and Its Ability, in Some Cases in Combination with a 5'-Nucleotidase Activity, to Generate Nucleosides from Extracellular Nucleotides and Nucleic Acids, the Protozoal Enzyme Functions in the Acquisition of Required Purines from Exogenous Sources (9, 10) Which Are Not Capable of Being Transported Across the Surface Membrane.** This enzymatic activity may therefore be essential to parasite replication and hence survival. In leishmanial parasites, which reside in the digestive tracts of sandfly vectors as well as in the phagolysosomal system of mammalian macrophages, the enzyme may enable the parasite to compete with its host cells for purines. As species of the genus Leishmania are important agents of human disease, this enzymatic activity may be a target for chemotherapeutic interventions for disease control. Indeed, purine metabolism has been recognized.
as an appropriate approach to controlling these organisms and purine analogs are being tested for their effectiveness (34).

A remarkable feature of the crithidial 3'-nucleotidase/nuclease, and an important area of further investigation, is the regulation of its activity. The availability of purified crithidial enzyme should allow for the development of suitable reagents for the further analysis of this system. Antibodies currently are being developed which should enable us, in conjunction with metabolic labeling, to determine if the increased enzymatic activity is due to the synthesis of new enzyme protein or if the increase results from modification of a pre-existing, catalytically inactive protein. Antibodies should also prove useful in screening libraries in efforts to clone the gene encoding the enzyme. The purified protein should also lead to the generation of amino acid sequences which will provide the basis for the production of synthetic oligonucleotide probes which will also allow us to analyze the regulatory events at the molecular level. Such developmental processes are critical for parasite survival as they allow the organisms to adapt to different host environments.

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