Plasmoidal transglutaminase of Physarum polycephalum was purified by anion exchange and hydrophobic chromatography. Gel filtration and SDS-polyacrylamide gel electrophoresis indicate that it is a monomer of 96–101 kDa. It is Ca\(^{2+}\)-dependent, with half-maximal activity at 0.7 mM Ca\(^{2+}\). Optimal activity occurs at pH 7.5 and at 50 mM KCl. Inactivation by N-ethylmaleimide indicates that it is a thiol enzyme. With N,N-dimethylcasein as substrate, the \(K_m\) for monodansylcadaverine is 33.9 ± 1.8 \(\mu\)M. Damage of plasmodia by brief treatment with 15% ethanol activates the transglutaminase, with rapid accumulation of cross-linked proteins unable to enter gels during SDS-polyacrylamide gel electrophoresis. Added monodansylcadaverine is conjugated principally to LAV1-2, a plasmodia-specific 40-kDa protein with four EF-hand sequences believed to bind Ca\(^{2+}\). Actin is seen as an additional substrate only in plasmoidal homogenates. Immunoblots show that upon ethanol treatment, a portion of LAV1-2 is modified quickly and shifts to 36 kDa; another portion is cross-linked to itself or other proteins. The modification of LAV1-2 may lead to localized release of Ca\(^{2+}\) and activation of transglutaminase for walling off damaged areas of plasmodia. No significant increase in amount of the transglutaminase occurs during starvation-induced differentiation of plasmodia to form spherules, but a 50% reduction in the amount of total protein leads to a doubling in the specific mass of the TGase. Neither the transglutaminase nor LAV1-2 is found in the ameboid form of the organism.

Transglutaminases (TGases)\(^1\) (EC 2.3.2.13) produce isopeptide cross-links between proteins by catalyzing acyl transfer between \(\gamma\)-carboxamide groups of glutamine residues and \(\varepsilon\)-amino groups of lysine residues. Alternatively, primary amino groups of polyamines such as cadaverine can serve as acceptors of the acyl group. TGases are found from bacteria to mammals, where they are present in various tissues and body fluids. Although the enzyme strictly requires Ca\(^{2+}\) in vertebrates and arthropods, this requirement does not extend to bacterial or apparently some plant TGases (for review, see Ref. 1).

Based on distribution and physical properties, mammalian TGases have been divided into five types. 1) Plasma TGase, factor XIII, is a tetramer of two catalytic \(\alpha\)-subunits and two noncatalytic \(\beta\)-subunits. During blood clotting factor XIII binds to polymerized fibrin, where it is activated to cross-link the fibrin by thrombin cleavage of the \(\alpha\)-subunits (2). A dimer of only the \(\alpha\)-subunits is present in the cytoplasm of some cells (3). 2) TGase I, keratinocyte TGase, is anchored to the cytoplasmic side of the plasma membrane through fatty acylation (4, 5). This enzyme is involved in terminal differentiation of keratinocytes through formation of a layer of cross-linked protein at the cell periphery (6). 3) TGase II, tissue TGase, is distributed in many tissues. It is primarily a cytosolic protein, but lesser amounts are found in membrane fractions and the extracellular matrix (7, 8). Although its full physiological role remains to be clarified, stabilization of extracellular matrices and cross-linking of intracellular proteins during apoptosis have been proposed as main functions of the TGase activity of the type II enzyme (9, 10). TGase II appears also to function as a G protein that mediates signal transduction from the \(\alpha\)-adrenergic receptor (11) and has GTPase and ATPase activities (12, 13). 4) TGase III, epidermal TGase, is expressed during terminal differentiation of epidermus and cells involved in hair formation. (14, 15). 5) TGase IV, prostate TGase, is a secreted homodimer (16) involved in the formation of copulatory plugs in rodents. Its physiological role in humans is unclear (17). The sequences of each of these types of TGase have been determined (1). All evolved from a common ancestor and show strong sequence conservation in a number of regions including that containing the acyl-accepting cysteine of the active site. This common ancestry is shared with the arthropod TGases of limulus hemocytes (18) and locust embryos (19). The bacterial TGase from Streptococci\(\text{ulum mobaarse}\) appears to have evolved independently (20) as have the filarial parasite TGases, which also have protein disulfide isomerase activity (21).

Little is known about TGases of lower eukaryotes. In the slime mold Physarum polycephalum, TGase activity was first indicated by the work of Lowey and Matacic (22). More recently, Klein et al. (23) reported the purification and partial characterization of a 40-kDa TGase from sclerotia of this organism. In its life cycle P. polycephalum alternates between ameboid and plasmoidal forms (24). The plasmodia are giant, multinucleated cells with a veined structure and no internal cell walls. In liquid shake-flask culture, macroplasmodia fragment into microplasmodia. Transfer of plasmodia to non-nutrient, salt-only medium causes reversible differentiation into dormant sclerotia, or spherules, with hard outer walls. Starvation of macroplasmodia together with exposure to light causes differentiation into sporangia that undergo meiosis to form haploid spores. Germinating spores form amebae, which can fuse to produce diploid plasmodia.

Because the TGase of P. polycephalum may play important roles in wound healing, envelope formation, and cellular stiffening during the life cycle and its study may provide insights into the function of tissue-type TGases, we have undertaken its further characterization. We report simplified methods for purification and assay of P. polycephalum TGase. Our results

\[\text{Characterization of 101-kDa Transglutaminase from Physarum polycephalum and Identification of LAV1-2 as Substrate}^*\]

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show that the active enzyme is a monomer with a molecular mass of about 100 kDa, present in both microplasmodium and spherules. It is a thiol enzyme, dependent on calcium, and is activated by ethanol damage of plasmodia. We identify the putative Ca \(^{2+}\)-binding protein LAV1-2 as a principal in situ substrate and propose a model for the function and activation of the enzyme.

**MATERIALS AND METHODS**

**Purification of TGase**—The following method, derived from Brookhart et al. (29), permitted purification of TGase within 8 h. *P. polycephalum* Mac with a doubly outlined of the central isolate Wisconsin I (26), was used. Microplasmodia were cultured in the semidefined liquid medium of Daniel and Baldwin (27) except that FeSO\(_4\) was substituted for FeCl\(_3\). Cultures were grown at 27 °C in the dark with shaking and harvested during exponential growth. Cells were packed by centrifugation at 100 × g for 5 min and washed with 0.4% glycerol, 20 mM potassium citrate, 10 mM KPO\(_4\), pH 5.0. After suspension in 2.5-ml volumes of KTE buffer (80 mM KCl, 20 mM Tris-HCl, 5 mM \(\beta\)-mercaptoethanol, 0.2 mM phenylmethylsulfonyl fluoride, pH 8.8), the cells were homogenized in a Waring blender at low speed using three 15-s pulses. The homogenate was centrifuged at 10,000 × g for 40 min. The resultant S-100 supernatant was passed slowly through the column. After centrifugation and washed as described for TGase purification. Pellets of microplasmodia (100 μl) in 1.5-ml microcentrifuge tubes were suspended in 400 μl of TM buffer (40 mM KCl, 10 mM NaCl, 7 mM MgCl\(_2\), 20 mM Tris-HCl, 1.4% glycerol, pH 7.5) containing 1.5 mM MDC at 23 °C. Ethanol was added to a final concentration of 15%; and after 30 s of mixing, the ethanol concentration was lowered to 5% by the addition of TM buffer. In a control experiment ethanol was initially added to a concentration of 5% through a 30-s period and then increased to 15% over a 30-s period and then increased to 15% over a 30-s period before washing and ethanol were removed at intervals and analyzed by 10.5% SDS-PAGE (31), with visualization of incorporated MDC using a UV lightbox. Gels were then stained with Coomassie Blue.

**Antibodies and Immunoblots**—CD-1 female mice (Charles River Laboratories) were immunized against plasmodial TGase inactivated by treatment with NEF. Freund's complete adjuvant and 100–200 μg of TGase were used for the first injection. Booster doses contained Freund's incomplete adjuvant and half as much TGase. Rabbit anti-actin (Sigma) was raised against the conserved COOH-terminal fragment, SGSIPVRKCF.

The TGase used for immunization was purified by a modified version of the method given above. The streptomycin-treated S-100 fraction was made 2 mM CaCl\(_2\) and applied directly to the phenyl-Sepharose column. After washing, the TGase was eluted with 80 mM KCl, 20 mM Tris-HCl, 2 mM dithiothreitol, 0.5 mM CaCl\(_2\), 10% glycerol, pH 7.9, for equilibration and elution. SDS-PAGE with silver staining of the purified TGase was used to confirm the purity of the TGase preparation. MDC was inactivated by adding NEM to 2.5 mM and incubating the mixture at 23 °C for 10 min. \(\beta\)-Mercaptoethanol was added to 5 mM, and the buffer was exchanged for Tris-buffered saline (25 mM Tris-HCl, 150 mM NaCl, pH 7.4) using a Centricon-30 concentrator.

For Western blots, proteins were separated by 10.5% SDS-PAGE and electoblotted onto nitrocellulose membrane using 12.5 mM Tris and 96 mM glycine as running buffer, and 25 mM Tris and 192 mM glycine as transfer buffer. After electrophoresis, the blots were treated with 5 m M KH\(_2\)PO\(_4\), 4 mM CaCl\(_2\), pH 5.1, to initiate differentiation to spherules (29). Incubation with shaking at 27 °C in the dark was resumed, and samples were removed for analysis at intervals over the next 45 h. The TGase mass was determined from Western blots of total sample proteins. For TGase enzymatic activity, the samples were suspended in KTE buffer, frozen with liquid N\(_2\), and ground to a powder in a mortar and pestle. After thawing, debris was removed by centrifugation at 15,000 × g for 15 min, and the supernatant was assayed.

**TGase Kinetic Studies**—TGase activity was assayed by fluorometric measurement of monodansylcadaverine (MDC) conjugation to N,N-dimethylcaseinate (DMC; 30). Standard reactions contained 2.5 mg/ml DMC, 0.5 mM MDC, 80 mM KCl, 10 mM Tris-HCl, 10 mM BisTris-HCl, 2 mM dithiothreitol, 2 mM CaCl\(_2\), pH 7.5, and 20–100 μg of purified enzyme in 100 μl. Incubation was at 23 °C for 30 min. For kinetic studies, the concentration of MDC ranged from 15 to 2,000 μM, and the reaction was followed for 40 min. Reactions were stopped by the addition of 200 μl of aceton and chilled on ice for 20 min. Precipitated protein was collected by centrifugation at 5,000 × g for 5 min, with cold 50% acetone, and solubilized in 400 μl of 0.1 M NaOH at 70 °C. The amount of incorporated MDC was determined by measuring the fluorescence of the solubilized protein using a Hitachi MPF-2A fluorescence spectrophotometer with an excitation wavelength of 326 nm, emission wavelength of 526 nm, and a 10-nm slit. The instrument was calibrated each time with MDC in 0.1 M NaOH.

**NEM Inhibition Assay**—To test for inhibition by NEM, a plasmoidal homogenate was made without \(\beta\)-mercaptoethanol. After centrifugation and washing as described for TGase purification, pellets of microplasmodia (100 μl) in 1.5-ml microcentrifuge tubes were suspended in 400 μl of TM buffer (40 mM KCl, 10 mM NaCl, 7 mM MgCl\(_2\), 20 mM Tris-HCl, 1.4% glycerol, pH 7.5) containing 1.5 mM MDC at 23 °C. Ethanol was added to a final concentration of 15%; and after 30 s of mixing, the ethanol concentration was lowered to 5% by the addition of TM buffer. In a control experiment ethanol was initially added to a concentration of 5% through a 30-s period and then increased to 15% over a 30-s period before washing and ethanol were removed at intervals and analyzed by 10.5% SDS-PAGE (31), with visualization of incorporated MDC using a UV lightbox. Gels were then stained with Coomassie Blue.

**Immunore screening of cDNA Library and DNA Sequencing**—Poly(A)\(^{+}\) mRNA was isolated from exponentially growing microplasmodia using a Total RNA Separator Kit (CLONTECH) and Poly(A)Ttract mRNA Isolation Kit II (Promega) and used to generate a unidirectional cDNA expression library in λZAPII (Stratagene) according to the manufacturers' protocols. Before insertion between the EcoRI and Xhol sites of the vector, cDNAs were size fractionated by gel filtration; cDNAs larger than 3.5 kb were cloned. The library, which contained 1 × 10\(^9\) recombinants, was screened with mouse anti-TGase serum at a 1:2,000 dilution by standard methods (32). Membranes were processed, and bound antibody was detected as described for Western blots. Positive recombinants were plaque purified, and cDNAs were recovered on pBlueScript SK\(^{-}\) by in vivo excision and transduction of Escherichia coli XLI-Blue (33).
For DNA sequencing, plasmid DNA was prepared using a QIAprep-spin plasmid kit (QIAGEN) and the manufacturer's protocol. Sequencing by the Sanger method and 32P labeling were performed using a T7 Sequenase sequencing kit (Amersham Pharmacia Biotech).

**Purification of (His)_6-LAV1-2 Fusion Protein**—LAV1-2 cDNAs were excised with PstI and KpnI from plasmids recovered by screening the cdNA library with anti-TGase antiserum and inserted between the PstI and KpnI sites of pRSET-A (Invitrogen). This construction replaced the Met-Ser dipeptide at the NH₂-terminus of LAV1-2 with a 38-residue peptide containing a (His)₆ sequence. After transformation of *E. coli* BL21(DE3)(pLysE), expression of the fusion protein was induced by the addition of 0.5 mM isopropyl-1-thio-

**Fluorometric determination of the amount of MDC cross-linked to DMC was used to monitor TGase activity. A summary of the purification steps used in the purification is given in Table I.**

### RESULTS

**TGase Purification**—Starting with the S-100 fraction of a homogenate of *P. polycephalum* microplasmodia, a TGase was purified by 300-fold with recovery of 50% of the initial activity. Fluorometric determination of the amount of MDC cross-linked to DMC was used to monitor TGase activity. A summary of the four steps used in the purification is given in Table I. After an initial streptomycin sulfate precipitation, the S-100 fraction was passed through a DEAE-cellulose anion exchange column. The unbound proteins were then loaded on a phenyl-Sepharose column in the presence of Ca²⁺ and eluted with EDTA. The fusion protein was dialyzed against 80 mM KCl, 20 mM Tris-HCl, 0.5 mM CaCl₂, 4% glycerol, pH 7.5, and stored at −20 °C. When tested as a substrate of TGase, it replaced DMC in the standard reaction.

**TGase Characterization**—Dependence of the TGase on Ca²⁺ was absolute, with half-maximal activity occurring at 0.7 mM Ca²⁺ (Fig. 3). Maximal activity was achieved at about 2 mM Ca²⁺. Optimum pH for the enzyme was 7.5, with activity falling by more than 50% 2 pH units below and above this range (Fig. 4A). The optimum KCl concentration was 50 mM, but activity of the TGase was more than 85% of maximum over the range of 0–100 mM KCl (Fig. 4B). Above 100 mM KCl, TGase activity declined steadily, reaching 45% at 400 mM. When assayed at increasing temperature, TGase activity peaked at 37 °C and declined to about 20% of maximal activity at 51 °C (Table II). Under optimum reaction conditions and saturating concentrations of the glutamyl donor DMC, the *Kₘ* for MDC, the acceptor amine, was 33.9 ± 1.8 μM. Above 0.5 mM DMC, the enzyme was partially inhibited. The TGase was irreversibly inhibited by NEM (Fig. 5), indicating the presence of a thiol group in its active site. This irreversible inhibition was completely dependent on the presence of Ca²⁺ and increased with increasing Ca²⁺ concentration. Inhibition to about 50% was achieved in a 7.5-min reaction at 1 mM Ca²⁺, similar to the concentration required for half-maximal TGase activity. Thus, the binding of Ca²⁺ to the TGase would appear to change its conformation, exposing the thiol of the active site to the NEM.

**Identification of an Endogenous 40-kDa Substrate**—To identify endogenous TGase substrates, reactions were performed in which MDC was added 1) to microplasmodia permeabilized by

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**Summary of TGase purification steps**

<table>
<thead>
<tr>
<th>Step</th>
<th>Total protein mg</th>
<th>Total activity units</th>
<th>Specific activity units/mg</th>
<th>Yield %</th>
</tr>
</thead>
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<td>122</td>
<td>4000</td>
<td>30.3</td>
<td>100</td>
</tr>
<tr>
<td>Streptomycin sulfate precipitation</td>
<td>129</td>
<td>4320</td>
<td>33.8</td>
<td>108</td>
</tr>
<tr>
<td>DEAE-cellulose chromatography</td>
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<td>2880</td>
<td>61.9</td>
<td>72</td>
</tr>
<tr>
<td>Phenyl-Sepharose chromatography</td>
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<td>2024</td>
<td>8800</td>
<td>51</td>
</tr>
</tbody>
</table>

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**FIG. 1.** Analysis of fractions during TGase purification by SDS-PAGE with Coomassie blue-staining (panel A) and immunoblotting with anti-TGase diluted 1:5,000 (panel B). Lane 1, plasmodial proteins; lane 2, S-100 fraction of plasmodial homogenate; lane 3, S-100 fraction after streptomycin sulfate precipitation; lane 4, proteins in flow-through from DEAE-cellulose column; lane 5, proteins unbound to phenyl-Sepharose column; lane 6, wash from phenyl-Sepharose column; lane 7, TGase eluted from phenyl-Sepharose column; lane 8, 10-fold concentrated sample of purified TGase. In lanes 2–7, equal portions of the fractions were loaded to permit comparison of the relative amounts of TGase. In lane 1, one-third as much sample was loaded. Only the portion of the immunoblot which showed antibody binding is illustrated.
Activity of purified TGase was assayed in the standard reaction at 2 mM Ca^{2+}, 80 mM KCl, pH 7.5, as described under "Materials and Methods."

<table>
<thead>
<tr>
<th>Temperature</th>
<th>Relative activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>°C</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>0.12 ± 0.00</td>
</tr>
<tr>
<td>16</td>
<td>0.36 ± 0.03</td>
</tr>
<tr>
<td>23</td>
<td>0.56 ± 0.10</td>
</tr>
<tr>
<td>30</td>
<td>0.79 ± 0.03</td>
</tr>
<tr>
<td>37</td>
<td>1.00 ± 0.00</td>
</tr>
<tr>
<td>44</td>
<td>0.64 ± 0.18</td>
</tr>
<tr>
<td>51</td>
<td>0.22 ± 0.03</td>
</tr>
</tbody>
</table>

MDC was incorporated principally into a 40-kDa protein and into large cross-linked complexes that remained in the wells or accumulated at the top of the separation gel. With the S-100 fraction, the 40-kDa protein and large cross-linked complexes were also prominently labeled by the MDC, but a band at 45 kDa, identified later as actin by anti-actin binding, was labeled equally well. Furthermore, as labeling progressed in the S-100 fraction a background of numerous labeled proteins appeared, demonstrating a more promiscuous choice of substrates among the soluble proteins in the S-100 fraction compared with the ethanol-treated microplasmodia. When all proteins were visualized on a duplicate SDS-PAGE gel by Coomassie Blue staining (Fig. 6A), no change in the protein profile other than increasing accumulation of high molecular mass complexes was seen in these samples taken at 3, 6, and 15 min after initiation of ethanol treatment.

When treatment with 0.05% Triton X-100 was used to permeabilize microplasmodia and release endogenous Ca^{2+}, incorporation of MDC was similar to that seen with the ethanol treatment, again indicating that in situ TGase catalyzes isopeptide bond formation (data not shown).

Antibodies against the p40 Substrate—In the initial stages of this work mice were immunized with TGase purified not as described above but by direct affinity chromatography of the S-100 fraction on phenyl-Sepharose followed by gel filtration on Sephadex G-200. Under these conditions the Ca^{2+} that was added to the S-100 fraction for the phenyl-Sepharose chromatography resulted in cross-linking of endogenous TGase substrates, which in the above purification scheme are removed by anion exchange chromatography before the addition of Ca^{2+}. SDS-PAGE had suggested that the TGase used for immunization was pure, and at the 1:5,000 dilution of the antiserum used for the immunoblot shown in Fig. 1, only the 101-kDa TGase

the addition of ethanol to 15% for 30 s followed by its dilution to 5% and 2) along with 2 mM Ca^{2+} to the S-100 fraction used for the TGase purification. The ethanol treatment was used in an attempt to trigger in situ TGase activation via release of endogenous Ca^{2+} while maintaining TGase substrates at their normal cellular locations. At intervals samples were withdrawn from the reactions and analyzed by SDS-PAGE and gel fluorography (Fig. 6B). In the ethanol-treated microplasmodia,
A cDNA library in Plasmodium was screened with the antiserum to identify a good substrate for the TGase. The preferred substrate identified was a 40-kDa protein, p40. Further analysis showed that the p40 was structurally unrelated to the TGase.

To confirm that the p40 substrate of TGase is LAV1-2, the LAV1-2 cDNA was transferred to a pRSET T7 expression vector, and the resultant (His)_6-LAV1-2 fusion protein was purified for use in an immunoblot competition assay. The result showed that the His-tagged LAV1-2 could compete for the binding of anti-p40 to immobilized p40 as effectively as the purified p40 (cf. Figs. 7 and 8). Furthermore, the purified (His)_6-LAV1-2 was found to be a good substrate for the TGase.

**LAV1-2 Modification during TGase Activation**

To follow the fate of LAV1-2 upon activation of TGase, samples of microplasmodium were taken over a 6-min period after ethanol treatment and analyzed by SDS-PAGE and immunoblotting (Fig. 9B). Within 30 s after the addition of ethanol, nearly half of the LAV1-2 was modified, perhaps by cleavage, to produce a form that migrated at 36 kDa. This conversion can be seen on a Western blot of plasmodial proteins (Fig. 7). The signal from the p40 on the blot was eliminated by the presence of the soluble p40, but the signal from the TGase remained relatively unchanged. Therefore, the p40 seemed structurally unrelated to the TGase.

Having failed to detect an enzymatic or structural relationship between the p40 and TGase, we considered the possibility that the p40 was a lower molecular weight TGase. To see if the p40 and TGase carried epitopes in common, the partially purified p40 was tested for its ability to compete for the binding of anti-TGase antibodies to TGase on an immunoblot of plasmodial proteins (Fig. 7). The signal from the p40 on the blot was eliminated by the presence of the soluble p40, but the signal from the TGase remained relatively unchanged. Therefore, the p40 seemed structurally unrelated to the TGase.

**The p40 Substrate of TGase Is LAV1-2**

A P. polypephalum cDNA library in λZAPII was screened with the antiserum containing both anti-p40 and anti-TGase. Four positives were found among 30,000 recombinants screened. All four contained a 1.6-kilobase insert with the same restriction profile. DNA sequencing and a database search showed that the inserts encode LAV1-2, a plasmodia-specific 40.5-kDa protein (34, 35). Furthermore, the purified (His)_6-LAV1-2 was found to be a good substrate for the TGase.
Coomassie-stained gel as well (Fig. 9A). At the same time, cross-linked complexes, which were recognized by the antiserum, appeared toward the top of the gel and in the sample wells and increased in amount over the 6 min the reaction was followed. The addition of purified (His)_6-LAV1-2 as a competitor during the immunoblot reaction with anti-TGase showed that the cross-linked complexes contained LAV1-2 and not TGase, which remained at the 101 kDa position (Fig. 9B). A similar modification of LAV1-2 and cross-linking was observed upon treatment of microplasmodia with 0.05% Triton X-100 and upon a shift of microplasmodia to 44 °C (data not shown).

TGase during the Physarum Life Cycle—Because TGase specific activity in extracts of acetone powder preparations has been reported to increase during starvation-induced differentiation of microplasmodia to spherules (23), we followed the level of the 101-kDa TGase during this differentiation. Exponentially growing microplasmodia were transferred to buffer containing 4 mM Ca^{2+} to initiate the differentiation. Over a 63-h period, during which spherules formed, samples of the culture were removed and analyzed by SDS-PAGE and immunoblotting with anti-TGase preadsorbed against LAV1-2. The results showed that the amount of TGase remained relatively constant (Fig. 10B), whereas total protein mass decreased by 50% as measured by the amount of Coomassie staining of an SDS-PAGE gel (Fig. 10A). Some TGase degradation products were apparent on the immunoblot early in starvation, but no antibody binding to bands below 76 kDa was observed at any time. The decrease in the amount of LAV1-2 during spherulation was similar to that of total protein (Fig. 10B).

When the specific enzymatic activity of the TGase in clarified homogenates of the starved cells was examined, it was found to increase 10-fold over the 63-h period (Fig. 11). However, this increase was not caused by an accumulation of greater amounts of TGase but by an approximately 10-fold decrease in amount of total protein in the clarified homogenates (Fig. 11). The TGase remained soluble while the majority of protein mass became insoluble, unless denatured with SDS.

To examine whether the 101-kDa TGase could account for all TGase activity in the homogenates of starved cells, samples containing equal units of TGase activity were immunoblotted with anti-TGase diluted 1:1,000 to detect the amount of 101 kDa TGase present (panel B).
P. polypecephalum Transglutaminase

point. Thus, the 101-kDa TGase can account for all TGase activity in spherules.

To examine further the 101-kDa TGase during spherule formation, we purified the enzyme from a culture after 30 h of starvation, using the one-step phenyl-Sepharose chromatography procedure described for TGase purification for antibody production, and we compared its $K_m$ for MDC with that of TGase from exponentially growing plasmodia. The two $K_m$ values were identical. Finally, we checked for the presence of inhibitors or activators of TGase. In a standard assay, which contained 2 mM Ca$^{2+}$ and purified TGase, the addition of an aliquot of homogenate prepared from exponentially growing microplasmodia or from starved, differentiating microplasmodia had no effect on the activity of the purified TGase. The TGase activities of homogenate and purified enzyme were simply additive, suggesting the absence of effectors. Adding heat-inactivated homogenates likewise did not affect the activity of purified TGase.

To check for the presence of a TGase in P. polypecephalum amebae, clarified homogenates were checked for activity in the standard TGase reaction. No incorporation of MDC was observed. In addition, amebal proteins were probed on an immunoblot for the presence of proteins able to bind the antibodies against the 101-kDa plasmodial TGase as well as LAV1-2. Neither of these proteins nor homologs were detected (Fig. 10).

**DISCUSSION**

The fact that the enzyme we have purified from P. polypecephalum plasmodia can form a covalent intermediate with dimethylated casein and transfer the casein moiety to the primary amine MDC clearly demonstrates that the enzyme is a transglutaminase. This TGase appears similar to the TGases of vertebrates and arthropods. Its molecular mass of 96–101 kDa is only moderately higher than the 75–90-kDa range known for the TGases of these organisms (1). Likewise, it is Ca$^{2+}$-dependent, with half-maximal activity at 0.7 mM Ca$^{2+}$, compared with 0.2–3 mM Ca$^{2+}$ for the vertebrate and arthropod enzymes. It also shares an optimum pH range just above 7 and a cysteine in the active site, based on NEM inhibition. In work not presented here, we found that the P. polypecephalum TGase is inhibited by GTP, like the tissue TGases of vertebrates (37, 38).

Using ethanol damage of microplasmodia to activate the TGase and incorporation of fluorescent MDC to detect in situ glutamine-donating substrates, we have identified LAV1-2 as the preferred substrate. Purified LAV1-2 is an equally effective substrate for the TGase in vitro. The existence of LAV1-2 in P. polypecephalum had been predicted on the basis of systematic sequencing of clones in a plasmodia-specific cDNA library (35). LAV1-2 mRNA represents 2.4% of plasmoidal mRNA (34), and LAV1-2 protein appears to be a similarly frequent protein based on our Coomassie-stained SDS-PAGE. By comparison with known protein sequence motifs, LAV1-2 was deduced to contain four copies of the EF-hand sequence characteristic of the tissue TGases of higher eukaryotes, which are antigenically unrelated. A lack of structural relatedness would be surprising, however, because the two TGases have many similar properties. Both are Ca$^{2+}$-dependent. Both are inactivated by NEM. Both have similar $K_m$ values for primary amines: 34 mM MDC for the 101-kDa TGase versus 21 mM spermine, 32 mM spermine, and 49 mM putrescine for the 40-kDa TGase. Both use actin as a preferred substrate in vitro. Therefore, we believe the 40-kDa protein seen by SDS-PAGE by Klein et al. was a TGase degradation product or a protein that contaminated their TGase preparation.

The 77-kDa molecular mass that Klein et al. observed upon gel filtration of their native TGase may have represented the intact or nearly intact TGase monomer rather than a dimer of the 40-kDa protein as originally proposed. By comparison we have obtained a value of 96 kDa for the TGase monomer by gel filtration.

Transfer of microplasmodia to salt medium with no carbon or nitrogen sources causes a Ca$^{2+}$-dependent differentiation to spherules using existing cellular resources. Within a day at least 70% of plasmoidal proteins are degraded and replaced in part by new proteins (40). By 48 h the protein mass of a culture is half the starting value, and the microplasmodia have cleaved into spherules with layered walls of glycoprotein...
and proteoglycan (41). Because TGase II may be involved in apoptosis in higher eukaryotes and TGase I is known to catalyze cell envelope stiffening and cornification, it is reasonable to hypothesize that TGase may play an important role in sphero-
ule formation in P. polycephalum. The critical role of high Ca\(^{2+}\) concentrations for spheroide formation also would be consistent with active participation of TGase. Klei et al. (23) have reported that TGase specific activity increases 6-fold in acetone powder extracts of microplasmodia undergoing spheroide formation. We have seen a 10-fold increase in TGase specific activity in clarified homogenates. Analysis of total protein by SDS-
PAGE and immunoblots with anti-TGase indicate that these increases are caused by reductions in total protein extracted from acetone powders or released in clarified homogenates. The amount of TGase remains relatively constant. Based on the 50% drop in total protein in cultures induced to spherulate, specific mass of the TGase in spheres would be double that in microplasmodia.

When the TGase is purified, its specific activity is unchanged during the differentiation, and no effectors were detected in homogenates of differentiating microplasmodia under our standard assay conditions. These observations, however, do not eliminate TGase as a possibly important player in differentiation of plasmodia to spheres. It is clear that the TGase mass is doubled relative to other proteins, and its activity can be regulated by changes in Ca\(^{2+}\) levels. Also, we have observed negative regulation of the TGase by both adenine and guanine nucleotides in experiments not presented here.

Based on activation of the 101-kDa TGase in plasmodia damaged by ethanol, Triton X-100, or heat, we propose that the enzyme is involved in coagulation of damaged areas of plasmodia. This would protect the remaining portion of the organism from exposure to the damaging agent and prevent endoplasm loss through chemical or physical wounds. The suggestion that TGase is active at the site of physical damage is supported by an earlier report that SDS extraction of endoplasmic drops extracted at punctures of macroplasmodial veins leaves an insoluble fibrous protein network (42). LAV1-2 may serve both as substrate for TGase and as transducer of a signal generated in response to plasmoidal damage. Assuming that LAV1-2 is a Ca\(^{2+}\)-binding protein, its observed rapid modification may release Ca\(^{2+}\) to activate vicinal TGase molecules for cross-linking unmodified LAV1-2 molecules to other proteins in the area of damage. Assuming the damage were localized, the released Ca\(^{2+}\) would soon dissipate leaving the TGase inactive because of its high Ca\(^{2+}\) requirement, 0.7 mM Ca\(^{2+}\) for half-
maximal activity.

Analysis of LAV1-2 with the PROSITE (43) program of EX-
PASy at the University of Geneva, Switzerland, shows multiple sites for potential phosphorylation, adding to the possible complexity of this protein. The sequence SPYGQDE, which is present in the middle and near both ends of LAV1-2, matches the consensus site for serine phosphorylation by casein kinase II-type enzymes that are known to be present in P. polycephalum (44). These sites, which contain possible donor glutamines, may provide an additional means for regulation of the TGase reaction.

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