Identification and characterization of an activated 20S proteasome in *Trypanosoma brucei*

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Abstract **Recently, we have reported the isolation and purification of 20S proteasomes from both the procyclic and bloodstream forms of *Trypanosoma brucei*, but no 26S proteasome was identified under those experimental conditions (Hua et al., Mol. Biochem. Parasitol. (1996) 78, 33–46). Subsequent attempts to identify a 26S proteasome in *T. brucei* led to the discovery of another form of the 20S proteasome designated the activated 20S proteasome because it exhibited much higher peptidase activities than the original 20S proteasome on all the fluorogenic peptides tested, and it crossreacted with the rabbit antisera against the 20S proteasomes purified from *T. brucei*. The activated 20S proteasome can be isolated from both procyclic and bloodstream forms of *T. brucei* and has a slightly higher molecular weight than the 20S proteasome. It is stable in the absence of ATP but susceptible to elution through a DE52 column. Analysis of the activated 20S proteasome in SDS-PAGE showed the presence of all the subunit proteins from the 20S proteasome plus an extra protein with an estimated molecular mass of 26 kDa. This protein, designated PA26, is not a degradation product of the 20S proteasomal subunit proteins. It could be a homolog of the bovine PA28 and human 11S regulator protein which form complexes with the 20S proteasomes resulting in activation of their peptidase activities. This likelihood was confirmed in a reconstitution experiment in which PA26 separated from the proteasome by a DE52 column chromatography was reintroduced into the purified 20S proteasome, and resulted in the emergence of a new protein band with the same mobility and peptidase activities as the activated 20S proteasome in native polyacrylamide gel electrophoresis. The presence of an activated 20S proteasome rather than a homolog of the 26S proteasome in *T. brucei* suggests that PA26 may play an important role in regulating the proteasome-mediated protein degradations in trypanosomes.**

Key words: Activated 20S proteasome; 20S Proteasome; *Trypanosoma brucei*

1. Introduction

The ubiquitin-proteasome-mediated pathway constitutes the major nonlysosomal protein degradation in the cytosol and nucleus of eukaryotes [1]. It plays a major role in controlling cell proliferation [2,3], cell cycle progression [4,5], cell differentiation [6], and gene transcription [7]. Recent evidence also suggests an indispensable role of the ubiquitin-proteasome pathway in MHC class I antigen processing and presentation [8,9]. The 26S proteasome is an essential component of the highly regulated, ATP-dependent proteolysis, which catalyzes the rapid degradation of many proteins of important biological functions such as ornithine decarboxylase (ODC) [10], cell cycle regulatory proteins (e.g. the cyclins) [4,11], transcriptional regulators (e.g. IκB and NF-κB) [7], as well as the abnormal proteins [12], which may arise from mutation or post-translational damage. In most cases, the ATP-dependent proteolytic pathway is ubiquitin-dependent. The substrates need to be polyubiquitinated in the presence of the ubiquitin-conjugating enzymes E1, E2 and E3 before being degraded by the 26S proteasome [1,13]. However, ubiquitin-independent proteolysis also exists. The best-studied example is the short-lived enzyme ODC, which is a key enzyme in polyamine biosynthesis. Degradation of ODC in mammalian cells requires a complex formation between ODC and another protein, the antizyme, without involving ubiquitination [10].

The proteolytic core in a 26S proteasome is the multicatalytic protease (MCP) [14] otherwise known as the 20S proteasome [15]. It is a barrel-shaped particle of four stacked rings with two α-rings at the ends and two β-rings in the center. Each ring is composed of seven subunit proteins and a feature which is conserved from the prokaryotes to the eukaryotes [16]. However, the subunit compositions are subjected to dramatic modifications in the course of evolution from as simple as two types of subunits (α and β) in the archaebacterium *Thermoplasma acidophilum* [17] to as many as 7 different α- and 7 different β-subunits in higher eukaryotes [16]. The construction of *T. acidophilum* 20S proteasome in the $\alpha_7\beta_7\beta_7\alpha_7$ form was recently confirmed by X-ray crystallography [18]. Generally, the purified 20S proteasomes possess only low levels of peptidase activity without any proteolytic activity demonstrable in vitro [19].

Precise regulatory mechanisms have been evolved to allow the 26S proteasomes to exist in the cytosol and nucleus of the eukaryotes without general proteolytic damages to the cell [20]. A number of such regulatory proteins, including the activators [21–23] and the inhibitors [24–26] of the proteolytic function, have been identified. PA700 [22] is a 19S ATPase complex containing multiple members of a nucleotide-binding protein family [27–30] and associating with the 20S proteasome in an ATP-dependent manner to form the 26S proteasome. On the other hand, an ATP-independent activator protein, PA28 from bovine [21] or an 11S regulator from human [31], was identified. The purified PA28 is a complex of two

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Abbreviations: MCA, 4-methylcoumarin-7-amido; Ac, acetyl; Suc, succinyl; tosyl, tolylsulfonyl ($CH_3C_6H_4SO_2$); LLVY-MCA, Suc-Leu-Leu-Val-Tyr-MCA; AAF-MCA, Suc-Ala-Ala-Phe-MCA; IIW-MCA, Suc-Ile-Ile-Trp-MCA; PFR-MCA, Pro-Phe-Ala-MCA; GGR-MCA, Cbz-Gly-Gly-Arg-MCA; AFK-MCA, Suc-Ala-Phe-Lys-MCA; YVAD-MCA, Ac-Tyr-Val-Ala-Asp-MCA; PMSF, phenylmethylsulfonyl fluoride; TLCK, tosyl-lysine chloromethylketone; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; DTT, dithiothreitol; ODC, ornithine decarboxylase; ELISA, enzyme linked immunosorbent assay

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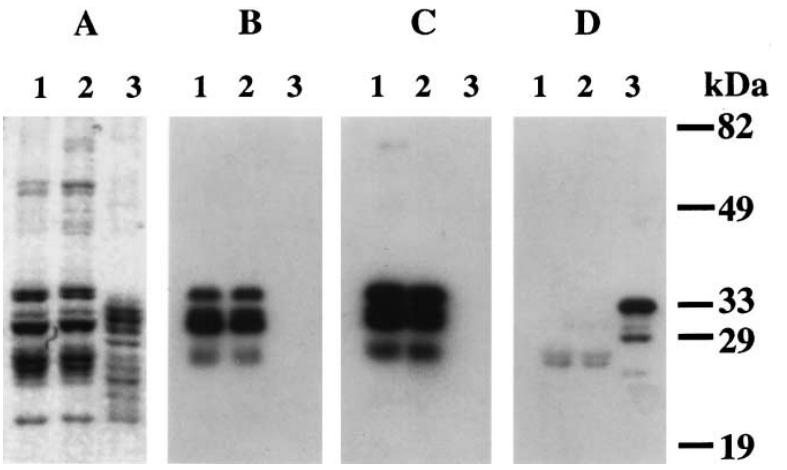

Fig. 1. SDS-PAGE and immunoblottings of the 20S proteasomes isolated from *T. brucei* and rat blood cells. Proteasomes were purified from the procyclic form (lane 1) and bloodstream form (lane 2) of *T. brucei* and rat blood cells (lane 3) using a previously described procedure [39]. The resolved proteasome subunit protein bands in 12.5% acrylamide gels were either stained with silver (A) or transferred to PVDF membrane and immunostained with the polyclonal antibodies against 20S proteasome from procyclic *T. brucei* (B), bloodstream *T. brucei* (C), and humans (D).

subunits, PA28α and PA28β, which share about 50% amino acid sequence identity [32,33]. They form a complex with the 20S proteasome and enhance its peptidase activities both in vitro [21,34,35] and in vivo [36,37]. The stoichiometry of this complex formation has recently been demonstrated as a heterohexamer composed of three PA28α and three PA28β subunits in an alternating order [36]. But its true biological function in vivo remains unknown. Recent evidence suggests a possible role for PA28 in regulating the quantity and quality of in vivo peptide production by the 20S proteasome for antigen presentation [13,38]. The in vivo interaction between the activated 20S and the 26S proteasomes to orchestrate the selective proteolysis remains to be explored.

Trypanosoma brucei is the causative agent of African sleeping sickness. This protozoan parasite can propagate as the procyclic form in the tsetse fly or differentiate into the bloodstream form in the mammalian host. Recently, we have identified and characterized the 20S proteasome from *T. brucei* [39]. Trypanosome 20S proteasomes exhibit striking morphological similarities to the rat blood cell 20S proteasome under electron microscopy. But the antibodies against the human 20S proteasome showed no crossreactivity to the trypanosome 20S proteasome. Furthermore, substrate specificity analysis showed that the trypanosome 20S proteasome has an unusually high trypsin-like activity, rather than chymotrypsin-like activity, which is the typical peptidase activity of rat 20S proteasome [39]. Leupeptin, an aldehyde known to inhibit the weak trypsin-like activity of mammalian proteasomes [40], demonstrated a potent inhibitory effect on the peptidase activity of purified trypanosome 20S proteasomes, further suggesting a possible functional dissimilarity between trypanosome and mammalian proteasomes. In fact, while the ODC-antizyme complex is subjected to rapid degradations by 26S proteasomes in mammalian cells, co-expression of mouse ODC and rat antizyme in *T. brucei* did not lead to rapid degradation of the former [41], suggesting that the trypanosomal proteasome may have evolved a catalytic property different from their mammalian host. The phenomenon became even more puzzling in our previous study when only a 20S proteasome, but not a 26S proteasome, could be purified from *T. brucei* in the presence of ATP and glycerol [39], which have been shown to stabilize the 26S proteasomes from rabbit reticulocytes, human kidney, rabbit skeletal muscle, *Drosophila* embryo, *Xenopus* oocytes, spinach leaves, and *Saccharomyces cerevisiae* [16]. In our numerous attempts to identify a 26S proteasome in *T. brucei*, the established purification procedure for 26S proteasomes was modified in different ways which led to the identification and characterization of another form of the 20S proteasome, which we called the activated 20S proteasome because it exhibited much higher peptidase activities than the original 20S proteasome. The activated 20S proteasome was identified and purified from both procyclic and bloodstream forms of *T. brucei*. It contains an extra protein of 26 kDa to the 20S proteasome. This 26 kDa protein can be separated from the proteasomes by DE52 column chromatography and re-introduced into the purified 20S proteasome to reconstitute the activated 20S proteasome. The presence of such an activated 20S proteasome rather than a homolog of the 26S proteasome in trypanosome suggests that the 26 kDa protein may play an important role in regulating the proteasome-mediated protein degradations in trypanosomes.

2. Materials and methods

2.1. Materials

Trypanosoma brucei strain 427 procyclic form and bloodstream form cells were prepared as described previously [39]. Rat blood cells were collected from Sprague-Dawley rats. Glycerol was from Fisher Scientific. The fluorogenic peptides LLVY-MAC, AAF-MAC, IIW-MAC, AFK-MAC, PFR-MAC, and GGR-MAC were purchased from Sigma, and YVAD-MAC from Peptide Internationals. Immobilon-P PVDF membrane was from Millipore. Molecular weight standards for SDS-PAGE were a group of broad range protein markers from New England Biolab. Prestained low molecular weight protein markers were from BioRad. The substrate for ELISA, *o*-phenyldiamine, was from Sigma. The rabbit antiserum against human 20S proteasome was a gift from Dr. Martin Rechsteiner (University of Utah). Horse radish peroxidase (HRP)-conjugated donkey antiserum against rabbit IgG was from Amersham.

2.2. Electrophoresis and immunoblotting

Native polyacrylamide gel electrophoresis (native PAGE) and SDS-PAGE were in 4.5% and 15% acrylamide, respectively, as described

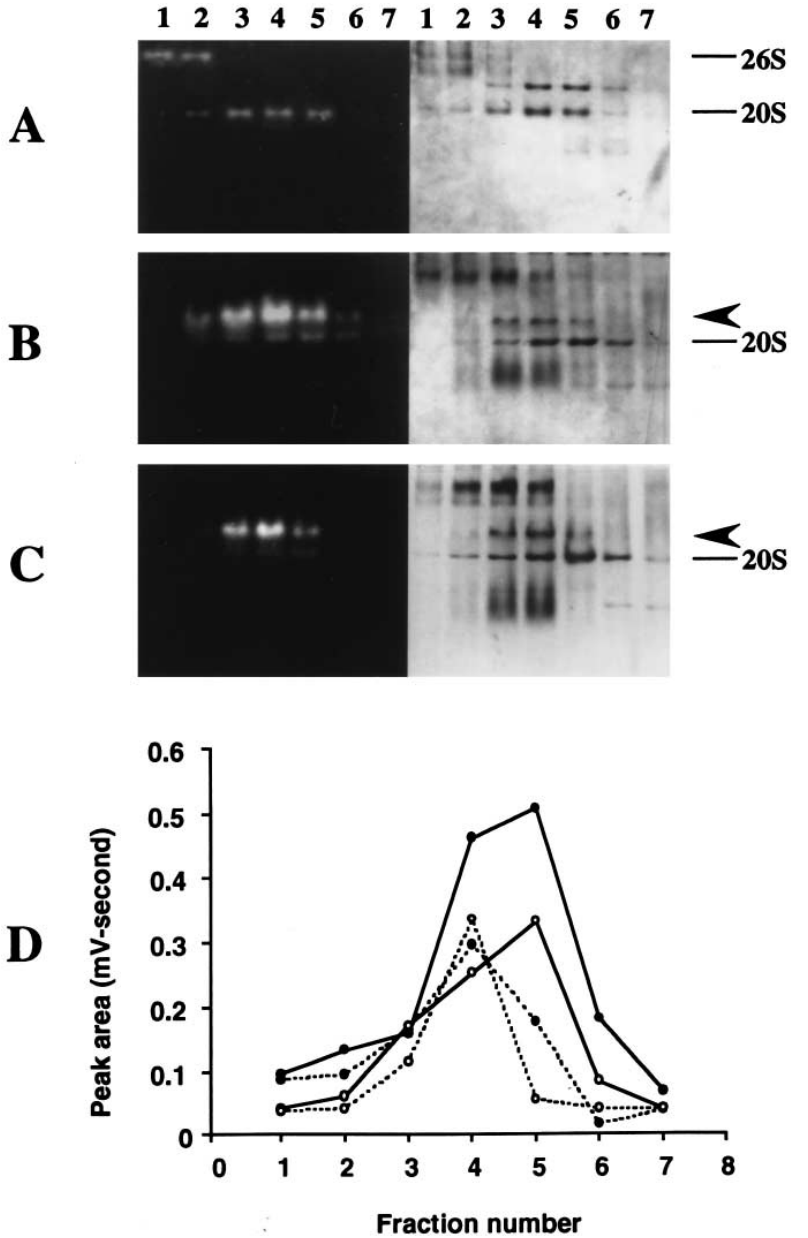

Fig. 2. Fractionation of proteasomes by glycerol gradient centrifugations. Crude proteasome fractions from rat blood cells (A), procyclic forms (B) and bloodstream forms (C) of *T. brucei* were further purified by glycerol gradient centrifugations (see Section 2). 10 μl of each collected fractions from the centrifugation was subjected to native PAGE and gel overlay assay using a mixture of LLVY-MCA, PFR-MCA and GGR-MCA as substrate. Numbers indicate the collected fractions (starting from the bottom of the tube) containing 50% (lanes 1–3), 40% (lanes 4–6) and 35% (lane 7) glycerol. Panels on the left are the gel overlay assay results, and those on the right are the same gels stained with Coomassie blue. The protein bands labelled with an arrowhead (upper band) and 20S (lower band) in panels B and C were traced with a densitometer and the peak areas covered by the upper bands (- - -) and lower bands (———) of panel B (●) and panel C (○) were plotted against the fraction numbers (D).

[39]. Immunoblottings were performed by a previously described procedure [39]. Densitometry scanning was performed with an LKB laser scanner.

2.3. Peptidase assay and gel overlay assay

Peptidase assays and gel overlay assays were performed as described previously [39]. The substrate used for the gel overlay assay was GGR-MCA unless otherwise specified.

2.4. Preparation of antibodies against the 20S proteasome from trypanosomes

The 20S proteasomes were isolated from the bloodstream forms and the procyclic forms of *T. brucei*, respectively, as previously reported [39]. The purified 20S proteasomes were further fractionated in native gel electrophoresis and the activity bands were excised from the gel for immunization of rabbit. Immunization and serum collection were performed by Animal Pharm Service, Inc., California. Specificity of the antisera was analyzed in immunoblottings of the purified 20S proteasomes resolved in SDS-PAGE.

2.5. Isolation of proteasomes from T. brucei

The entire purification procedure was modified from the procedures of Hua et al. [39] and carried out at 4°C unless otherwise noted. Briefly, 2 ml of freshly prepared cell pellets of *T. brucei* were washed twice with 10 ml phosphate-buffered saline containing 10% glucose, and used immediately for purification. Cell pellets were resuspended in

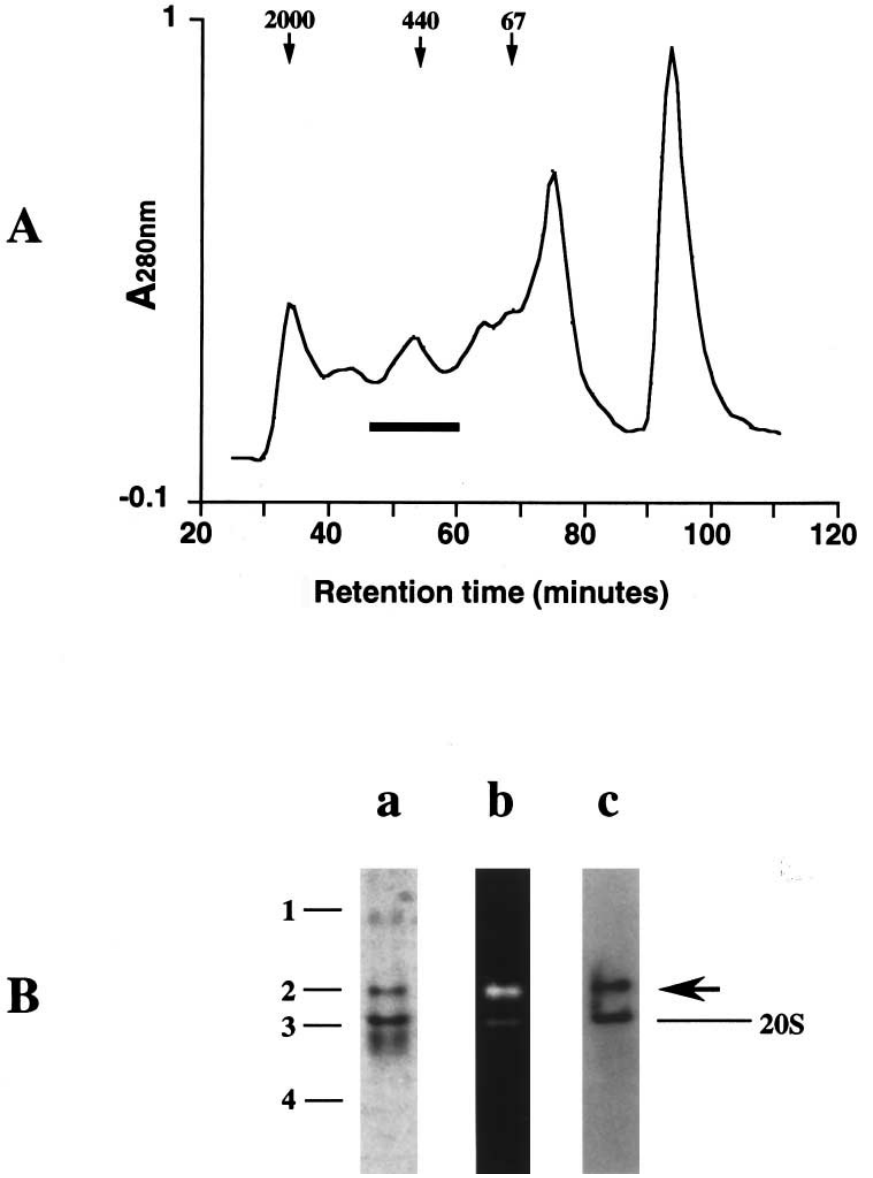

Fig. 3. Identification of the activated 20S proteasome from *T. brucei*. A: A crude proteasome fraction from procyclic form *T. brucei* cells was applied to a 1×30 cm column of Superose 6. Each collected fraction was analyzed by native PAGE and gel overlay assay to identify the fractions containing proteasomes, pooled (horizontal bar) and concentrated with an Amicon 30 concentrator. Arrows indicate the positions of molecular weight markers including dextran blue (2000 kDa), ferritin (440 kDa) and bovine serum albumin (67 kDa). B: The pooled and concentrated sample from A was analyzed in native PAGE stained with Coomassie blue (panel a); the gel overlay assay (panel b); and immunoblotting stained with the rabbit antiserum (panel c) to the purified 20S proteasomes from procyclic *T. brucei*. The arrowhead points to the activated 20S proteasome. The numbers 1, 2, 3 and 4 on the left designate the protein bands visualized by Coomassie blue staining.

two volumes of buffer A (10 mM Tris-HCl pH 7.4, 25 mM KCl, 10 mM NaCl, 1 mM $MgCl_2$, 0.2 mM EDTA, 1 mM DTT and 2 mM ATP) containing 1 mM TLCK, 1 mM PMSF, and 20% glycerol. Cells were lysed by sonication until no apparent intact cells were observed under the light microscope. The lysate was cleared by centrifugation at 80 000×*g* for 60 min. Proteasome fractions were recovered from the supernatant by ultracentrifugation at 100 000×*g* for 60 min in a Beckman TLA100 [39]. Protein pellets were resuspended in 200 μl buffer B (buffer A containing 1 mM TLCK, 1 mM PMSF, and 5% glycerol), and the insoluble material was removed by centrifugation at 80 000×*g* for 30 min. The cleared supernatant was referred to as the crude proteasome fraction.

The crude proteasome fraction (200 μl) was overlaid on top of a stepwise glycerol gradient (1.8 ml) containing 225 μl of 15, 20, 25, 30, 35, 40, 45, and 50% glycerol in buffer B, and centrifuged at 38 000 rpm with a TLS55 rotor (Beckman) for 16 h. Fractions (100 μl) were collected from the bottom of the tube. Peptidase activities in these fractions were immediately fractionated in native PAGE and stained by gel overlay assay followed by Coomassie blue staining.

2.6. Isolation of the activated 20S proteasome from T. brucei

The crude proteasome fraction was prepared from 5–7 ml trypanosome cell pellets in the absence of ATP, Millipore filtered and fractionated by gel filtration chromatography on a Superose 6 column equilibrated with buffer A at a flow rate of 0.2 ml/min. Fractions of 0.5 ml were collected, and the content of each fraction was analyzed in native PAGE and stained for peptidase activity followed by Coomassie blue stain. Fractions containing both the original 20S proteasome and the activated 20S proteasome were pooled with the glycerol concentration brought down to 5% and concentrated with Centricon 30 to a final volume of 200 μl. The sample was further fractionated in glycerol gradient centrifugation as described above, and the purified fractions were stored at −70°C.

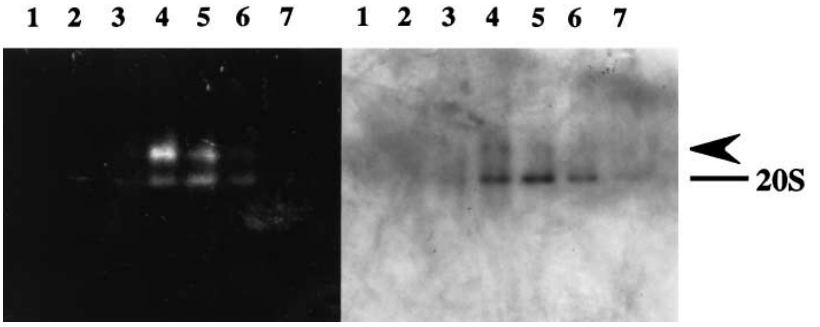

Fig. 4. Fractionation of proteasomes from the procyclic form of *T. brucei* in the absence of added ATP. The experimental protocols were identical to those detailed in Fig. 2 except that no ATP was added throughout the purification.

2.7. Separation of the 26 kDa protein from T. brucei proteasomes and its reconstitution with the 20S proteasome

Proteasome fractions containing both the 20S proteasome and the activated 20S proteasome were purified from the bloodstream form of *T. brucei* as described previously. The proteasome fraction (140 μg) was passed through a mini-column (BioRad) containing 200 μl DE52 equilibrated with buffer C (buffer A containing 20% glycerol without ATP). The column was then washed with 10 ml buffer C and step eluted with 300 μl elution buffer C containing 0.10, 0.15, 0.20, 0.30 and 0.40 M KCl. 10 μl of each of the eluted fractions was analyzed by SDS-PAGE followed by silver staining. The fractions eluted from 0.10 and 0.15 M KCl and containing the 26 kDa protein were pooled. The KCl concentration was brought down to 0.05 M by dilution with buffer C and concentrated to 50 μl using a Centriprep 30 (Amicon). The total protein was measured with the Bradford method and the purity of 26 kDa protein was analyzed in a densitometer.

The 20S proteasome from the bloodstream form was purified as described previously [39]. 4 μl (1252 ng) of this fraction was incubated with 20 μl 26 kDa protein (58.5 ng) at 37°C for 20 min. The sample was then analyzed in native PAGE. The peptidase activity was measured by gel overlay assay using a mixture of fluorogenic peptides containing GGR-MCA, LLVY-MCA and PFR-MCA. The protein bands were revealed by silver staining.

2.8. Measurements of specific peptidase activity

The original 20S proteasomes and the activated 20S proteasomes were separated from each other by a preparative native PAGE. The two activity bands were excised after gel overlay assay and incubated in an equal volume of buffer A for 1 h with occasional agitation. Supernatant was then used in the peptidase assays on different individual fluorogenic peptides and proteasome concentrations were determined by enzyme linked immunosorbent assays (ELISA) (see below). The specific peptidase activity of the purified proteasome was expressed as nmol of MCA generated by 1 mg protein within 1 min. Protein concentrations of the proteasome samples were estimated with the Bradford assay (BioRad). For those samples with protein concentrations too low to be detected by the Bradford method, the method of ELISA was used, carried out at 37°C unless otherwise specified. Briefly, proteasome samples were two-fold serially diluted in 0.1 M carbonate-bicarbonate buffer, pH 9.6, and added to each well (100 μl) in duplicate. A serially diluted 20S proteasome standard was included in each plate and processed in parallel with the sample. The plate was incubated for 1 h and non-specific bindings were blocked with phosphate buffered saline (PBS) containing 1% bovine serum albumin, pH 7.4. The plate was washed twice with PBS containing 0.05% Tween-20 (PBS-Tween). 100 μl rabbit antiserum against trypanosomal 20S proteasome (1:1000) was introduced and incubated for another hour. After washing thrice with PBS-Tween, 100 μl HRP-conjugated donkey anti-rabbit antiserum (1:5000) was added and incubated for 30 min. The plate was washed five times with PBS-Tween, and 100 μl freshly prepared 0.034% (w/v) substrate solution with 2% hydrogen peroxide in 0.2 M citrate-phosphate buffer pH 5.0 was added. After a 30 min incubation, the reaction was stopped by 100 μl 2 M sulfuric acid and the absorbance at 490 nm was measured using a Dynatech Microplate Reader with a reference filter at 600 nm. Concentrations of the proteasome samples were determined by referring their optical density to the standard curve.

3. Results

3.1. Antibodies to T. brucei 20S proteasomes do not cross-react with the 20S proteasome from rat blood cells

A rabbit antiserum to the purified 20S proteasome from bloodstream form *T. brucei* was tested in immunoblottings and shown to crossreact with proteasomes from the two forms of *T. brucei* but not with that from rat blood cells (Fig. 1C). Similarly, antibodies to the 20S proteasome from procyclic form *T. brucei* recognized the proteasomes from both forms of *T. brucei* but not that from rat blood cells (Fig. 1B). As shown previously [39], a rabbit antiserum to the human 20S proteasome crossreacted with the rat blood cell 20S proteasome but failed to react with most of the subunit proteins in the 20S proteasomes from the bloodstream and the procyclic forms of *T. brucei*, except for a doublet band in the 24 kDa region (Fig. 1D). These data indicate that the structures of subunit proteins in the 20S proteasome from *T. brucei* are mostly distinct from those in the rat 20S proteasome. The

Table 1
Comparisons of specific peptidase activities (nmol/mg/min) between the 20S and the activated 20S proteasomes purified from the bloodstream and procyclic forms of *T. brucei*

Substrate	Blood stream form			Procyclic form		
	Proteasomes		Activation factor	Proteasomes		Activation factor
	20S	Activated 20S		20S	Activated 20S	
LLVY-MCA	69.2	217.8	3.1	6.2	91.0	14.6
AAF-MCA	19.6	104.5	5.3	2.3	59.0	25.4
IIW-MCA	20.2	211.3	10.5	4.8	72.7	15.0
AFK-MCA	1.3	104.5	78.7	0.6	64.3	106.9
PFR-MCA	34.2	120.8	3.5	43.3	90.3	2.1
GGR-MCA	158.0	292.7	1.9	147.9	210.7	1.4
YVAD-MCA	0.8	88.9	112.7	0.4	69.7	156.6

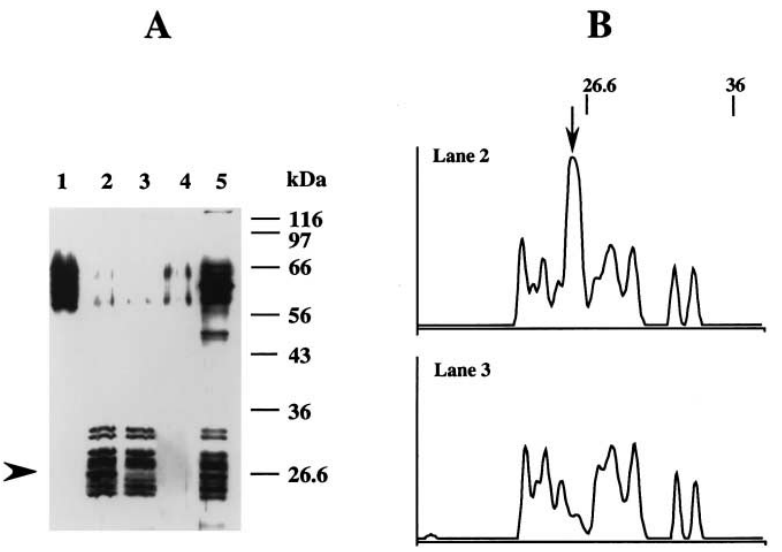

Fig. 5. SDS-PAGE analysis of the individual protein bands identified in the native PAGE of purified proteasomes from the procyclic form of *T. brucei*. The individual protein bands 1, 2, 3 and 4, identified in Fig. 3B panel a, were each excised from the native polyacrylamide gel and subjected to SDS-PAGE in lanes 1–4 (panel A). Lane 5 contains a sample of the crude proteasome fraction from the procyclic form *T. brucei* cells as described in Fig. 3. The silver-stained lanes 2 and 3 were each analyzed in densitometer tracings with the results presented in panel B.

proteasomes from the two forms of *T. brucei* differ very little (see Fig. 1B,C) except that the antibodies against bloodstream *T. brucei* proteasomes react a little more strongly with the subunit protein(s) in the 34 kDa region (Fig. 1C). There was weaker immunostaining of protein bands in the 24 kDa region by either preparation of antibodies, which may be attributed to varying immunogenicities of individual proteasomal subunit proteins and different immune responses in individual rabbits rather than a proteasomal subunit protein profile change between the two forms of *T. brucei*.

3.2. *No 26S proteasome can be identified or isolated from T. brucei*

When the proteasomes were isolated in the presence of added ATP (2 mM) in our previous efforts, both 20S and 26S proteasomes were obtained from the rat blood cells whereas only the 20S proteasomes were obtained from *T. brucei* [39]. A similar result was reproduced in the present isolation of proteasomes from rat blood cells. The 26S proteasome was clearly identified in the 50% glycerol fraction of the density gradient (Fig. 2A, lanes 1 and 2), well separated from the 20S proteasome sedimented in the 40% glycerol fraction (Fig. 2A, lanes 3, 4 and 5). However, when both forms of *T. brucei* were brought through this same purification procedure, no peptidase activity band corresponding to the 26S proteasome was detectable in the 50% glycerol fraction after the ultracentrifugation (Fig. 2B,C, lanes 1 and 2). Although a major protein band was observed in the 26S proteasome region in Fig. 2B,C, lanes 1–4, it contained only protein of 56–66 kDa but not the 20S proteasome subunit proteins in subsequent SDS-PAGE analysis (see Fig. 5A, lane 1). There are two possible explanations for this negative outcome: (a) *T. brucei* 26S proteasome becomes dissociated during this purification; (b) *T. brucei* does not have a 26S proteasome (see Section 4).

Upon a closer examination of Fig. 2B,C, however, we noticed the presence of two peptidase activity bands in the *T. brucei* sample from the 40% glycerol fraction (Fig. 2B,C, lanes 3–5). The lower band corresponds to the 20S proteasome purified from both forms of *T. brucei* in the previous study [39]. The upper band appears to consist of less protein than the corresponding 20S band but much higher peptidase activity. An analysis of the intensity of each Coomassie blue stained protein band versus the location of each collected fraction in the glycerol gradient centrifugation (Fig. 2D) indicates that the upper band has moved a little farther toward the bottom of the gradient than the 20S proteasome, suggesting a somewhat higher molecular weight than the *T. brucei* 20S proteasome. Similar results were reproduced in each of the 6 independent purification experiments performed subsequently, suggesting that this small discrepancy in molecular weights is real (data not shown). Comparisons between the individual integrated protein peak areas in Fig. 2D show that the ratio between the upper and lower bands is 0.51:1 in the procyclic form (Fig. 2B) and 0.67:1 in the bloodstream form of *T. brucei* (Fig. 2C). An analysis of the individual activity bands by densitometer tracings indicates that the ratio between the upper and lower bands is 12:1 in the procyclic form (Fig. 2B) and 73:1 in the bloodstream form of *T. brucei* (Fig. 2C). The upper band in the bloodstream form thus appears to have 4.6 times higher specific peptidase activity than that in the procyclic form on a combination of substrates LLVY-MCA, PFR-MCA and GGR-MCA.

3.3. *Identification of an activated 20S proteasome species in T. brucei*

The crude proteasome fractions prepared from both the bloodstream and the procyclic forms of *T. brucei* (see Section 2) were each fractionated through a Superose 6 (Pharmacia) gel filtration column, and the fractions containing a broad protein peak in the estimated range of 440 kDa were pooled (Fig. 3A). This pooled sample demonstrated two major protein bands in native PAGE (Fig. 3B, lane a) corresponding to the upper protein band and the 20S proteasome band in Fig. 2B,C. Gel overlay assay for peptidase activity confirmed the previous observation that the upper protein band possesses a

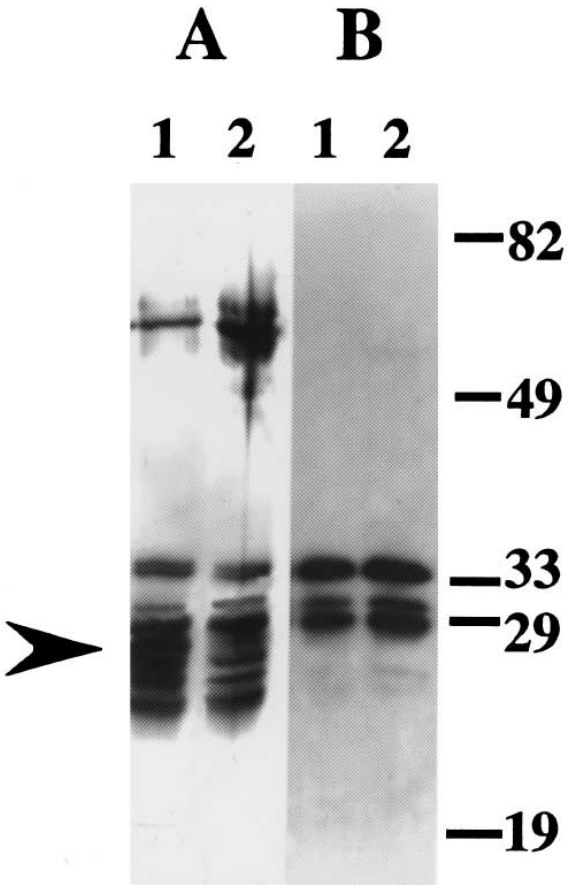

Fig. 6. SDS-PAGE (A) and immunoblottings (B) of the purified activated 20S proteasome (lane 1) and the purified 20S proteasome (lane 2) from the procyclic forms of *T. brucei*. The experimental details are the same as described in Fig. 1. The antibodies used were from a 1:1 mixture of rabbit antisera against the purified 20S proteasomes from both forms of *T. brucei*.

much higher peptidase activity than the 20S proteasome (Fig. 3B, lane b). Immunoblotting of the same native PAGE gel with the rabbit antiserum to the 20S proteasome from procyclic *T. brucei* had both protein bands stained by the antibodies (Fig. 3B, lane c). This upper protein band must thus contain some or all of the subunit proteins from *T. brucei* 20S proteasome.

The upper protein band and the 20S proteasome band were each separately excised from the native gel and eluted into buffer solution. They were then each assayed on a variety of fluorogenic peptide substrates for construction of the profiles of their peptidase activities. The results presented in Table 1 demonstrate that the peptidase activity profiles of the 20S proteasomes from both forms of *T. brucei* are very similar to each other and essentially the same as reported from our previous studies [39]. Thus, they have very high peptidase activity on Suc-GGR-MCA but rather low activities on Suc-YVAD-MCA and Suc-AFK-MCA. The specific activities between the two extremes differ by more than two orders of magnitude (Table 1). When the eluted upper protein band was examined in the same assays, it demonstrated higher peptidase activities on all the substrates and achieved a similarly high specific activity range of 64.3 to 292.7 nmol/mg/min on all the fluorogenic peptides tested (Table 1). These assays were repeated on 4 different batches of proteasomes purified from both forms of *T. brucei*. The absolute values of the specific peptidase activities in the purified samples varied from batch to batch, depending on the quality of each purified sample. But activities in the upper protein band remain uniformly high on all substrates and, consequently, the factor of activation between the upper protein band and the corresponding 20S proteasome band on each substrate stays relatively unchanged among different batches of proteasomes (data not shown). We thus designated the upper protein band the 'activated 20S proteasome'.

3.4. Characterization of the activated 20S proteasome from *T. brucei*

In our subsequent studies, ATP was eliminated from the purification procedure (see Section 2). The result turned out to be the same, i.e. both the 20S proteasome and the activated 20S proteasome were isolated from *T. brucei* in a similar ratio as previously indicated (Fig. 4). The stability of the activated 20S proteasome is thus apparently independent of ATP. A recent review of our previous efforts in purifying proteasomes from *T. brucei* [39] indicated that the activated 20S proteasome was never detected either in the presence or in the absence of 2 mM ATP. There was, however, one extra step in the previous purification procedure that was eliminated from the present procedure, the proteasomes were used to be adsorbed to a DEAE-cellulose column (DE52) and eluted in a gradient of 50–300 mM KCl in buffer A containing 2 mM ATP [39]. When a mixture of the 20S proteasome and the activated 20S proteasome from our present study were eluted through the DE52 column in 300 mM KCl, the activated 20S proteasome could not be recovered, thus suggesting an instability of the activated 20S proteasome at relatively high ionic strengths.

3.5. Identification and characterization of a 26 kDa protein from the activated 20S proteasome

The independence from ATP and the sensitivity toward high salts have been among the characteristics of the bovine PA28 activator protein-20S proteasome complex [21]. This complex consists of a 20S proteasome associated with a hexamer of the 28 kDa activator protein PA28 at either end of the cylindrical structure [36]. In order to verify whether the activated 20S proteasome from *T. brucei* represents a similar complex, the individual protein bands 1, 2, 3 and 4 from the native PAGE of *T. brucei* proteasomes were each excised from the gel (Fig. 3B, lane a) and subjected to SDS-PAGE (Fig. 5A). The results show that the only detectable difference between the purified activated 20S proteasome and the purified 20S proteasome (compare lanes 2 and 3) is that the former consists of an extra major protein band with an estimated molecular mass of 26 kDa. Densitometer tracing of the two lanes (Nos. 2 and 3) indicated an approximate ratio of 12:1 between the 26 kDa protein band and one of the well-separated 32 kDa subunit protein band (Fig. 5B). Although the result may suggest an association of about 12 molecules of the 26 kDa protein with each *T. brucei* 20S proteasome, the variation in silver staining efficiency of different proteins abrogates this conclusion in the absence of other supporting data. We then tried to verify whether the 26 kDa protein could be a degradation product from one of the 20S proteasome subunit proteins as exemplified in the case of proteasomes from human erythrocyte [40] (see Section 4). Rabbit antisera against

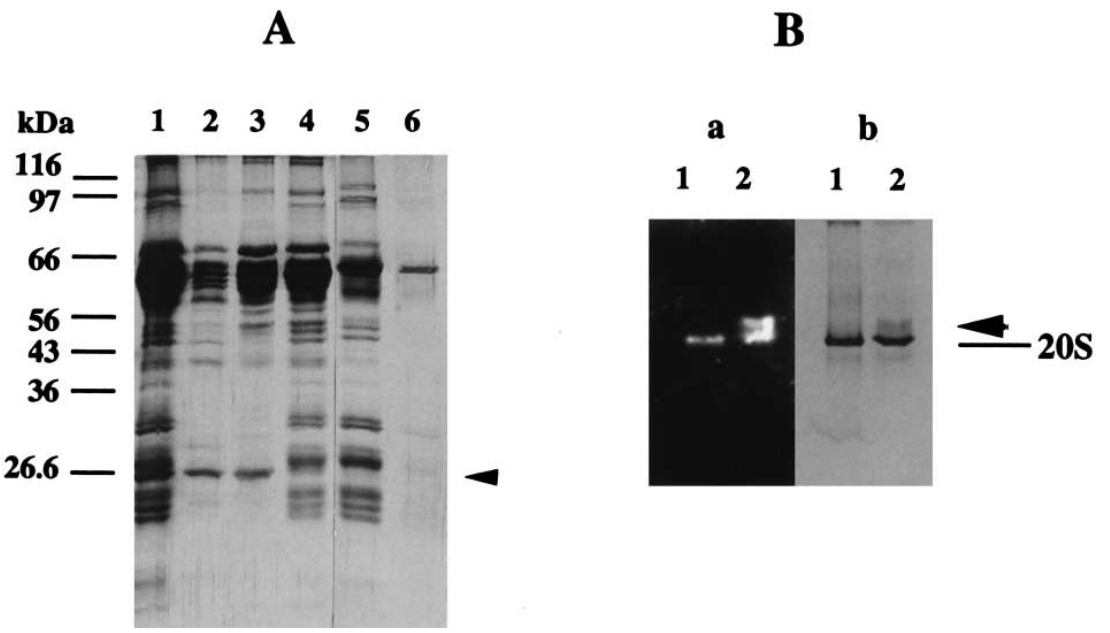

Fig. 7. Separation of the 26 kDa protein from the proteasomes from *T. brucei* bloodstream forms and reconstitution of the activated 20S proteasome. A: SDS-PAGE analysis of the fractions collected from a DE52 column chromatography of a crude proteasome sample; lane 1, the crude proteasome sample; lane 2–6, fractions collected from 0.10 M, 0.15 M, 0.20 M, 0.30 M and 0.40 M KCl, respectively. B: Native PAGE analysis of the incubated mixture of the 26 kDa protein and the 20S proteasome. Panel a, the silver stains. Lane 1, the 20S proteasome from *T. brucei* bloodstream forms; lane 2, the incubated mixture. The arrowhead in panel A indicates the band of 26 kDa protein; the arrow in panel B points to the activated 20S proteasome.

the purified 20S proteasomes from both forms of *T. brucei* were mixed in an equal ratio and tested on immunoblottings of the gels from SDS-PAGE of the activated 20S proteasome from *T. brucei.* The results, shown in Fig. 6, suggest that the 26 kDa protein is not a degradation product of the 28–34 kDa subunit proteins stained prominently by the antibodies.

We then separated the 26 kDa protein from the total proteasome fraction isolated from the bloodstream form of *T. brucei* by DE52 column chromatography. The fractions eluted from the column were analyzed in SDS-PAGE (Fig. 7A). The results demonstrate that the 26 kDa protein was eluted exclusively at 0.10 M and 0.15 M KCl (Fig. 7A, lanes 2 and 3, respectively) without any indication of contamination from the 20S proteasome. The latter was eluted at 0.20 and 0.30 M KCl (Fig. 7A, lanes 4 and 5, respectively) which exhibit a typical profile of the subunit proteins of *T. brucei* 20S proteasome without the presence of the 26 kDa protein band.

The isolated 26 kDa protein sample was desalted and combined with the 20S proteasome purified from *T. brucei* bloodstream forms at an approximate molar ratio of 1.4:1. The mixture was incubated at 37°C for 20 min and analyzed in native PAGE for protein and peptidase activity (Fig. 7B). The results indicated the emergence of a faint protein band with a slower mobility than the 20S proteasome from the reconstitution experiment (Fig. 7B, panel b, lane 2). Although the slower-moving band has much less protein than the 20S proteasome band, they share roughly equivalent levels of peptidase activities (Fig. 7B, panel a, lane 2), thus suggesting that the former is most likely the activated 20S proteasome.

The positive outcome from the reconstitution experiment have provided a strong evidence that the 26 kDa protein is a homolog of bovine PA28 capable of forming a complex with the 20S proteasome in trypanosome to bring the latter to an activated state. We thus designated the 26 kDa protein 'PA26' in the subsequent discussion.

4. Discussion

In our present investigations, an 'activated' 20S proteasome species was identified and isolated from both bloodstream and procyclic forms of *T. brucei.* The activated 20S proteasome is apparently a complex between our originally identified 20S proteasome [39] and a 26 kDa protein PA26. Although further analysis of PA26 has not yet been carried out to see if it represents more than a single molecular species, the independence from ATP, the instability in passing through a DE52 column and the lack of immune crossreactivity between PA26, the other 20S proteasome subunit proteins exhibited by this activated 20S proteasome and the outcome from the reconstitution study suggest that it may be similar to the structure of bovine PA28-20S proteasome complex [21].

The specific peptidase activities in the activated 20S proteasome are uniformly higher on all the fluorogenic peptides tested thus far and attain similarly high levels, regardless of whether the proteasomes are of bloodstream form or procyclic form origin. The lack of substrate specificity has negated the highly unusual substrate specificities originally observed with the regular 20S proteasome, i.e. very high trypsin-like activity, low chymotrypsin-like activity and extremely low peptidylglutamyl-peptide hydrolase activity [39]. Similar observations on the mammalian proteasomes indicated that binding of PA28 or 11S regulator to the 20S proteasomes also markedly altered the profiles of substrate specificity of the latter [21]. With the non-discriminatory enhancements of peptidase activities on all peptides tested, it is anticipated that the profile of protein degradations in *T. brucei* will also change with the changing

ratios between the activated and the regular 20S proteasomes in this organism. Since *T. brucei* is known to lack vigorous transcriptional regulation of its gene expression [42], it will be interesting to raise the question whether a regulation of the ratio between the activated and the regular 20S proteasome in *T. brucei* plays a role in controlling gene expression and cellular differentiation. The expression of PA28 and 11S regulator in mammalian cells is induced by interferon γ [32,43]. The latter is also found in our recent studies to induce a MAP kinase homolog in bloodstream *T. brucei* but not in the procyclic form of *T. brucei* [44]. It remains to be seen whether interferon γ will also induce the PA26 in the bloodstream *T. brucei* and thus cause a changed profile of protein degradations from that of the procyclic form. The higher ratio of the activated vs. regular 20S proteasomes in the bloodstream form (Fig. 2D) tends to support this possibility, though many more experimental data will be needed to verify the biological function of the activated 20S proteasome inside *T. brucei*.

Mammalian 20S proteasomes have been known to be activatable by heating to 55°C or incubating with basic polypeptides, SDS, guanidine HCl or fatty acids; or even dialysis against water [45–47]. But we do not consider the activated 20S proteasome from *T. brucei* a result from any of the conditions mentioned above, because it was not exposed to any of them during its isolation. In the 20S proteasome purified from human erythrocytes, the proteolytic cleavage of a 32 kDa subunit protein to a 28 kDa protein was found correlated with the stimulation of proteasomal activity [48]. But the PA26 in the activated 20S proteasome from *T. brucei* did not crossreact with the antibodies to the purified proteasome, and is thus unlikely to be a degradative product from any one of the proteasomal subunit proteins (see Fig. 6). PA26 is more likely a homolog of bovine PA28 [21] and human 11S regulator [31] because (a) its association with the 20S proteasome is not affected by ATP (see Figs. 2 and 4); (b) it can be separated from the 20S proteasome by elution through a DE52 column with 0.10 M to 0.15 M KCl (see Fig. 7A); (c) it can be combined with the 20S proteasome to reconstitute the activated 20S proteasome (see Fig. 7B). It is thus reasonable to conclude that the PA26-20S proteasome complex is the activated proteasome from *T. brucei*.

The apparent absence of a 26S proteasome from *T. brucei* under the present experimental conditions is puzzling but we cannot conclude from the data that the 26S complex does not exist in this organism. Since two polyubiquitin homolog genes have been identified in *T. brucei* [49], and ubiquitinated proteins have been detected in the high molecular weight regions of the *T. brucei* crude extracts [50], it has been assumed that the ubiquitin-proteasome protein degradation pathway is functional in *T. brucei*, thus suggesting that the 26S proteasomes are present in this organism. However, recent studies have indicated that ubiquitination of IκB in T-lymphocytes activates its kinase activity instead of causing its degradation [51]. Ubiquitination is thus not always associated with the function of 26S proteasomes. Other recent studies in our laboratory have also provided additional evidence indicating that two highly conserved protein components, S4 and S5a, in the 19S complex of 26S proteasomes are missing from the crude extracts of *T. brucei* bloodstream and procyclic forms (unpublished). S4 is one of the six putative ATPases found in the 26S proteasomes from yeast [52] and mammals [53]. There is 75% sequence identity between yeast and human S4, and the latter can function in place of its yeast homolog within the 26S complex [52]. S5a binds specifically to multi-ubiquitinated polypeptide chains with high affinity [54], and homologous genes exist in many organisms [55]. Rabbit polyclonal antibodies to human S4 and S5a, obtained from Dr. Martin Rechsteiner of the University of Utah, were tested in immunoblottings of the *T. brucei* crude extracts, and no crossreacting protein band was detected in the extracts of either bloodstream or procyclic forms of *T. brucei* (data not shown). Although negative results are usually difficult to interpret, it is tempting to postulate that *T. brucei* may depend primarily on a balance between the 'activated' 20S proteasome and the regular 20S proteasome for controlled protein degradations.

The ubiquitins have been identified only among the eukaryotes [56]. The archaebacterium *T. acidophilum* possesses only simple 20S proteasomes [57], whereas *Escherichia coli* has the large multimeric proteases La [58] and Clp [59] both linking ATPase activity and proteolysis together without ubiquitin-dependent protein degradation. A 26S proteasome species was identified in *S. cerevisiae* [60], but no PA28 homolog has been described in the yeast thus far. The mammals have apparently acquired both the 26S proteasome and the PA28-20S proteasome complexes [61]. Now that our present data suggest that the trypanosomes may have only the 20S and the PA26-20S proteasomes, it may cast some new light on the course of evolution of proteasomes from the prokaryote to the eukaryote kingdom.

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