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# Studies on tsetse midgut factors that induce differentiation of bloodstream *Trypanosoma brucei brucei* in vitro

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**Abstract.** An in vitro system for studying the transformation of bloodstream forms of *Trypanosoma brucei brucei* into procylic (midgut) forms is described. In this system, transformation of the parasites was stimulated by *Glossina morsitans morsitans* midgut homogenates at 27° C but not at 4° C. The transformation-stimulating capacity was irreversibly destroyed by heating the midgut homogenates at 60° C for 1 h. A correlation was established between the transformation activity of the midgut homogenates and trypsin activity. The protease inhibitors (soybean trypsin inhibitor and *N-p*-tosyl-L-lysine-chloromethyl-ketone) inhibited trypsin activity and completely blocked the transformation of the parasites. Furthermore, the midgut homogenates could induce transformation only in the presence of blood. These results provide evidence for the involvement of trypsin or trypsin-like enzymes within the tsetse midgut in stimulation of the transformation of bloodstream trypanosomes.

During the life cycle of African trypanosomes, the parasites undergo various developmental changes within the mammalian host and the tsetse fly vector (Hoare 1972). Within the vector, these changes start immediately upon ingestion of an infected bloodmeal and result in the formation of procyclics from the bloodstream forms of the parasite (Vickerman 1965; Vickerman and Preston 1976; Englund et al. 1982). This process involves a series of complex morphological and physiological changes that enable the parasites to adapt to a radically different environment within the fly midgut. For example, the transformation process is accompanied by loss of the surface coat, cessation of synthesis of the variable surface glycoprotein (VSG), activation of mitochondrial enzymes and the cytochrome electron-transport system, induction of procyclin synthesis, and displacement of the positions of the nucleus and kinetoplast (Vickerman and Preston 1976; Barry and Vickerman 1979; Ghiotto et al. 1979; Roditi and Pearson 1990). Since transformation is an important first step in the establishment of infection and subsequent transmission, elucidation of the factors involved in triggering the process has been the subject of many recent investigations (Bienen et al. 1980, 1981, 1983; Overath et al. 1983; Durieux et al. 1991).

Studies on *Trypanosoma congolense* showed that pronase treatment of the parasites led to dramatic changes, including removal of the surface coat, displacement of the nucleus and kinetoplast and reticulation of the mitochondria (Frevert et al. 1986). These changes resembled those associated with differentiation of bloodstream trypanosomes into midgut forms. Other studies have described various systems that induce transformation of trypanosomes in culture. For example, *cis*-aconitate and temperature reduction have been reported to stimulate the transformation of monomorphic *T. brucei* in vitro (Brun and Schonenberger 1981; Simpson et al. 1985; Czichos et al. 1986). In *T. cruzi*, a factor present in the midgut of *Triatoma infestans* triggers differentiation of the epimastigotes (Isola et al. 1986). Recent studies have shown that treatment of monomorphic culture forms of *T. brucei* with trypsin provide the signal for transformation in vitro (Yabu and Takayanagi 1988). However, the mechanisms underlying this process are not presently clear. In this report, we describe an in vitro system for studying the transformation of bloodstream *T. b. brucei* into procyclic forms and provide evidence supporting a role for midgut trypsin in the induction of the process.

## Materials and methods

### *Experimental insects and animals*

Tsetse flies *Glossina morsitans morsitans* Westwood were supplied by the Insect Mass Rearing Technology Unit of the International Center of Insect Physiology and Ecology (ICIPE). Male rats (Wistar strains) and New Zealand white rabbits were used. Pleomorphic *Trypanosoma brucei brucei* of a stock derived from EATRO 1969 was used. This stock was isolated as previously de-

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scribed (Otieno et al. 1983). The parasites were originally grown in rats from stabilates that had been cryopreserved in liquid nitrogen. Parasitised blood was obtained from the rats by cardiac puncture before the peak of parasitaemia. The parasites were isolated using a Percoll gradient (Grab and Bwayo 1982) and were suspended in phosphate saline glucose (0.15 M phosphate and 0.1 M NaCl containing 10% glucose). The parasites were counted using a haemocytometer equipped with improved Neubauer ruling.

### *Transformation assays*

In the in vitro assay system, 50–60 teneral flies (24 h after emergence) were allowed to feed on a rat for 30 min. The flies were immobilised by brief chilling, after which the midguts were carefully dissected. The midguts were then teased gently and mixed with freshly isolated parasites or parasitised blood. Unless otherwise stated, all incubations of such mixtures were carried out at 27° C. At different times, the incubation mixture was vortexed and 20-µl aliquots were withdrawn for the preparation of wet smears. In the in vivo studies, flies were fed on parasitised blood and the midguts were dissected at various times. The wet smears were fixed in methanol, stained with Giemsa's stain and examined using a Dialux compound microscope (Leitz Wetzlar, FRG). Typically, 3–4 groups of 100 parasites each were counted and classified as being typical bloodstream forms, midgut forms or transition forms on the basis of morphological characteristics (Lloyd and Johnson 1924; Ghiotto et al. 1979).

### *Enzyme assays*

Trypsin activity was assayed using a chromogenic substrate, carbobenzoxy-val-gly-arg-4-nitranilide acetate (Chromozym-TRY; Boehringer-Mannheim, FRG). The reaction mixtures contained Chromozym-TRY (40 µmol) and either crude midgut homogenates or bovine pancreas trypsin dissolved in 50 mM TRIS/HCl (pH 8). The reactions were initiated by the addition of substrate and the increase in absorbance at 410 nm was monitored using a Beckman model DU 50 spectrophotometer (Palo Alto, Calif., USA) fitted with a thermostat control. The change in molar extinction at 410 nm ($\varepsilon_{410}=8{,}800$; Erlanger et al. 1961) was used to determine the amount of substrate hydrolysed; 1 unit trypsin is the amount of enzyme required to hydrolyse 1 µmol Chromozym-TRY $min^{-1}$ (30° C).

### *Effect of temperature on transformation*

In this experiment, the transformation assays were carried out exactly as described above, except that the incubations were conducted at 4° C for 6 h and at 27° C for a further 6 h. In another experiment, the midguts from previously fed flies were heated in a water bath and the transformation assays were carried out at 27° C using parasitised blood. Trypsin assays were also performed at various intervals during the incubation period.

### *Effect of inhibitors on transformation and trypsin activity*

In this study, increasing concentrations (0–1 µg/ml) of soybean trypsin inhibitor (STI; Millipore Corp., Freehad, USA) were added to midgut homogenates prior to the enzyme assays. In a parallel experiment, STI (1 µg/ml) was added to midguts from previously fed flies and the transformation assays were carried out as described above. A similar experiment was performed in vivo except that the flies were fed via an artificial silicone membrane on parasitised blood containing the inhibitor. In this case, the flies were killed at hourly intervals and the transformed parasites were assessed as described above. The same experiments were repeated using *N-p*-tosyl-lysine-chloromethyl-ketone (TLCK, 0–0.12 mg/ml; Sigma, St. Louis, Mo., USA).

### *Conditions required for trypsin release*

In this experiment, midguts were dissected from unfed flies and then one group was teased gently, whereas the other was homogenised for 1 min using a Virtis homogeniser (Gardiner, USA). Trypsin activity was determined and transformation assays were carried out in each case. In a related experiment, trypsin and transformation activities were measured using midguts obtained from flies that had previously been fed on phosphate saline glucose through a membrane.

### *Effect of trypanosomes on enzyme activity*

In this study, freshly isolated trypanosomes were added to either gently teased or homogenised unfed midguts and then incubated (6 h, 27° C). At hourly intervals, aliquots were withdrawn for determination of trypsin activity. The experiment was similarly repeated using increasing concentrations of the trypanosomes ($10–10^6$/ml). The effect of trypsin on the parasites was also examined. In this case, the parasites were incubated (6 h, 27° C) with increasing amounts of midgut homogenates representing different trypsin concentrations. After 6 h, the stained parasites were examined microscopically.

## Results

### *Time course of the transformation process*

The time course for the in vitro transformation of trypanosomes from bloodstream to procyclic forms in the presence of midgut homogenates is shown in Fig. 1. After approximately 5 h, 50% of the bloodstream parasites had transformed into either transition or midgut forms at 27° C. No bloodstream forms were detectable after 12–14 h, and the midgut forms were first detectable after approx. 4–5 h. By 20–22 h, all of the parasites had transformed into midgut forms. In the in vivo assays, in which midguts were dissected at different intervals from flies that had previously fed on parasitised blood, the transformation process followed the same general trend, with 50% of the bloodstream parasites having transformed after 6 h incubation (Fig. 1). There was no apparent significant difference in transformation rates observed in the two assays. No transformation was observed in the absence of the midgut homogenates (data not shown).

### *Effect of temperature on transformation and enzyme activity*

The results of this experiment showed that the parasites did not transform when the incubation mixture was maintained at 4° C (Fig. 2). However, as the temperature was raised to 27° C, a gradual stimulation of the transformation process was observed. Similarly, the trypsin

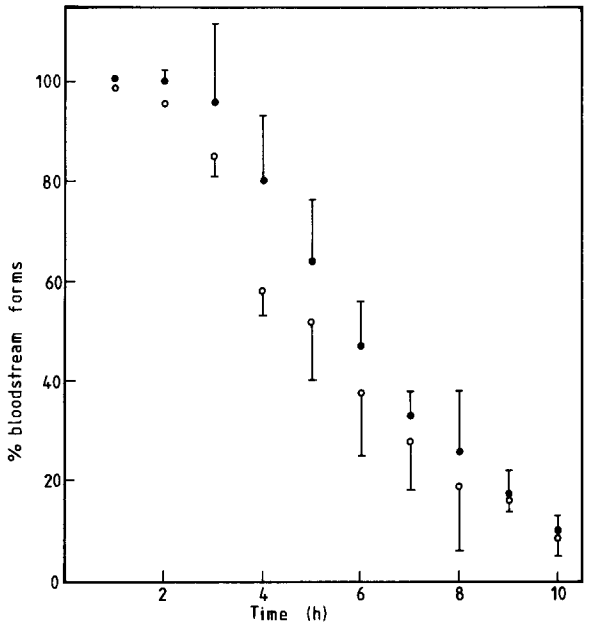


**Fig. 1.** Time course of the transformation process. In the in vivo assay (●), *Glossina morsitans morsitans* were fed on infected rat. At different intervals, flies were killed and the midguts were dissected. In the in vitro assay (○), midgut homogenates from 50 previously fed flies were incubated with trypanosomes ($10^6$/ml). In both cases, transformation of the parasites was assessed as described in Materials and methods. Data represent mean values ± SD ($n=5$)

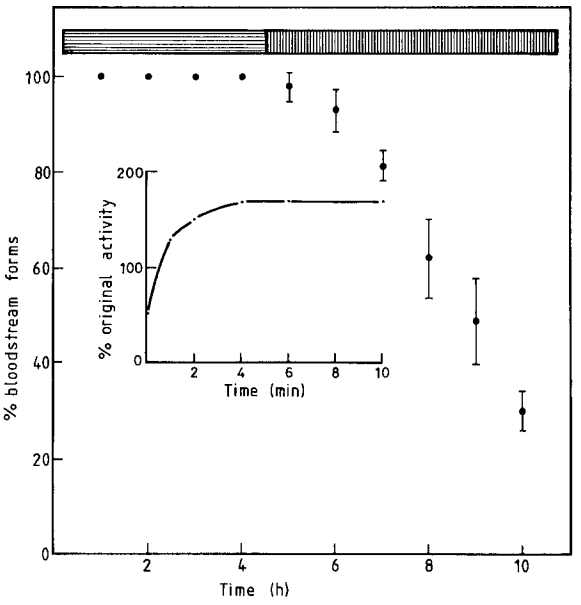


**Fig. 2.** Effect of temperature on transformation and trypsin activity. Midgut homogenates from previously fed flies were mixed with parasites as described in Fig. 1 and incubated for 5 h each at 4° C (▤) and 27° C (▥). Transformation of the parasites was assessed as described in Materials and methods. *Inset:* Trypsin activities were determined in midgut homogenate at 4° C and at different times (0–10 min) as the temperature was raised to 27° C. Results (% enzyme activity before the temperature was lowered to 4° C) represent the mean values for 3 determinations

activity in midgut homogenates was low at 4° C ($\sim 1.5 \times 10^{-2}$ units). However, as the temperature was raised to 27° C, a rapid stimulation of enzyme activity occurred, reaching a plateau after 4 min. This represented an increase of >100% as compared with the activity noted before the temperature was lowered to 4° C (Fig. 2, inset).

*Effect of heat treatment on transformation and enzyme activity*

In this study, the effect on transformation of heating the midgut homogenates at 60° C for 1 h was examined. The results showed that this treatment completely destroyed the ability of the homogenates to transform bloodstream trypanosomes (Fig. 3). Trypsin activity was also destroyed under similar conditions, but a 10% residual enzyme activity remained after the treatment (Fig. 3, inset).

*Effects of protease inhibitors on trypsin activity and transformation*

The effects of two protease inhibitors, STI and TLCK, on the transformation of trypanosomes and on trypsin activity were assessed as described in Materials and methods. The results showed that STI ($\sim 1$ µg/ml) completely inhibited transformation under the in vitro conditions used (Fig. 4). However, the same relative concentration of STI did not completely block the process under in vivo conditions, and approximately 20% of the parasites had transformed by 10 h (Fig. 4). Similarly, 90% of the midgut trypsin activity was inhibited by approx. 1 µg/ml STI, and the 10% residual trypsin activity observed was probably due to other proteases that are not sensitive to this inhibitor (Fig. 4, inset). Similar effects were observed using TLCK, except that a concentration of 0.12 mg/ml was required to reduce trypsin activity by 90% and to block the transformation process.

*Inhibition of midgut trypsin activity by trypanosomes*

Homogenisation of 50 fly midguts resulted in the release of $\sim 8 \times 10^{-2}$ units trypsin as compared with $\sim 2 \times 10^{-2}$ units in the gently teased and tapped midguts (Table 1). However, when parasitised blood was added, a marked initial decrease in trypsin activity was observed in both cases after 1–2 h incubation (Table 1). Thereafter, the enzyme activities remained relatively constant for the rest of the incubation period. The effect of the trypanosomes on midgut trypsin activity was found to be concentration-dependent (data not shown).

*Stimulation of transformation by midgut homogenates and bovine pancreas trypsin*

The ability of midgut and pancreatic trypsin to induce transformation of bloodstream trypanosomes in vitro

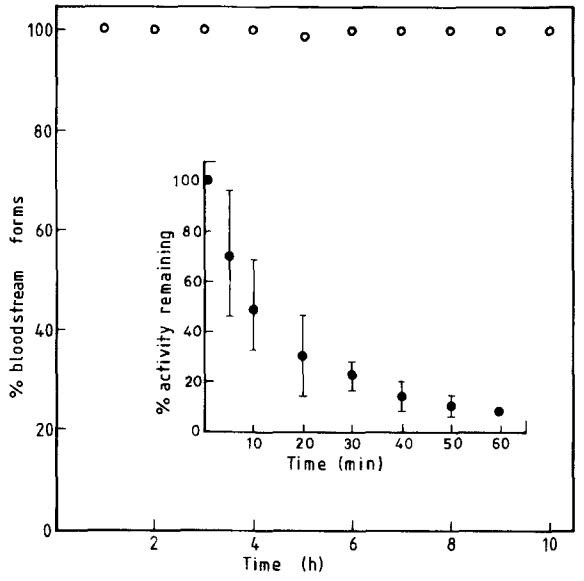


**Fig. 3.** Effect of heat treatment on transformation and midgut trypsin activity. Midgut homogenates from previously fed flies were heated in a water bath (1 h, 60° C) and then incubated with parasitised blood ($10^6$ trypanosomes/ml). At various intervals, the transformed parasites (○) were assessed as described in Materials and methods. *Inset:* The effect of heat treatment on enzyme activity. Trypsin was assayed in midgut homogenates heated at 60° C for various periods (0–60 min). Data (expressed as % of control activity at 27° C) represent mean values ± SD ($n=4$)

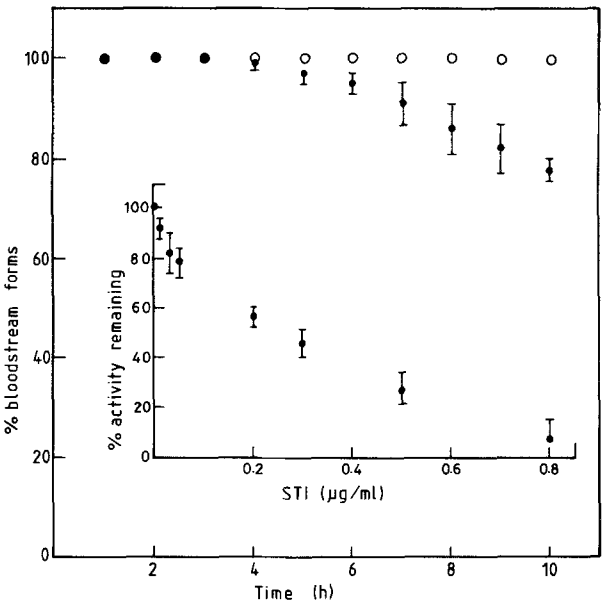


**Fig. 4.** Effect of STI on transformation and trypsin activity. Midgut homogenates from previously fed flies were mixed with STI (~ 1 µg/ml) and then incubated with parasitised blood ($10^6$ trypanosomes/ml) (○). Under in vivo conditions, flies were fed on parasitised blood that contained ~1 µg/STI/ml (●). In both cases, the parasites transformed were assessed at various intervals. Data represent mean values ± SD ($n=4$). *Inset:* Increasing concentrations of STI (0–0.8 µg/ml) were mixed with midgut homogenates prior to the enzyme assays. Data (expressed as % control activity at 27° C) represent mean values ± SD ($n=4$)

**Table 1.** Inhibition of midgut trypsin by trypanosomes in vitro

| Time (h) | 0 | 1 | 2 | 3 | 4 | 5 | 6 |
|---|---|---|---|---|---|---|---|
| Tapped midguts | 2.1 ± 0.05 | 1.6 ± 0.07 | 0.6 ± 0.02 | 0.8 ± 0.1 | 0.6 ± 0.09 | 0.4 ± 0.05 | 0.5 ± 0.09 |
| Homogenised midguts | 7.9 ± 0.09 | 3.4 ± 0.01 | 2.9 ± 0.08 | 3.6 ± 0.2 | 3 ± 0.5 | 3 ± 0.07 | 2.9 ± 0.4 |

Midguts dissected from 50 unfed flies were either teased or homogenised and then incubated (6 h) with parasitised blood ($10^6$ trypanosomes/ml). Enzyme activities (units × $10^{-2}$) were determined as described in Materials and methods. Data represent mean values ± SD ($n=4$)

**Table 2.** Stimulation of transformation by trypsin in vitro

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Midgut trypsin | 0 | 0.4 | 1.2 | 1.5 | 2.7 | 2.9 | 3.5 | 4.9 | 5.8 |
| Midgut forms (%) | 0 | 13 ± 2.9 | 20 ± 0.6 | 27 ± 0.6 | 42 ± 2.9 | 46 ± 5.1 | 26 ± 5.5 | 9 ± 1.6 | – |
| Pancreas trypsin | 0 | 0.6 | 1.1 | 1.9 | 4 | 4.9 | 5.7 | 6.1 | 7.2 |
| Midgut forms (%) | 0 | 5 ± 2.4 | 9 ± 0.9 | 12 ± 1.5 | 21 ± 1.5 | 27 ± 3 | 10 ± 4 | – | – |

Parasitised blood ($10^6$ trypanosomes/ml) was incubated (27° C for 6 h) with various concentrations of either midgut or bovine pancreas trypsin (expressed as units × $10^{-2}$). The transformed parasites were assessed as described in Materials and methods. Data represent mean values ± SD ($n=4$)

was examined. The results showed that ~3 × $10^{-2}$ units midgut trypsin transformed approximately 50% of the bloodstream trypanosomes after 6 h incubation in the presence of blood (Table 2). The same trypsin concentration was ineffective in inducing transformation in the absence of blood (data not shown). The process could similarly be stimulated using bovine pancreas trypsin, except that much higher concentrations were required and a smaller number of trypanosomes were transformed (Table 2).

## Discussion

This report describes an in vitro system for the transformation of bloodstream trypanosomes into the procyclic

(midgut) forms. Using this system, we have presented evidence for the involvement of tsetse fly midgut trypsin or trypsin-like enzymes in the transformation of bloodstream trypanosomes. Under in vitro conditions, the process could also be stimulated by commercial trypsin. In either case, the process proceeded efficiently only in the presence of optimal enzyme concentrations and of whole blood.

Initial evidence for the presence of a midgut factor that stimulates the transformation of bloodstream trypanosomes was obtained by incubating the parasitised blood with midgut homogenates from tsetse flies. Although a drop in temperature from 37° to 27° C has been reported to stimulate the differentiation of *Trypanosoma brucei* culture forms in vitro (Simpson et al. 1985; Czichos et al. 1986; Kaminsky et al. 1988), the freshly isolated bloodstream parasites used in the present experiments did not transform in the absence of midgut homogenates, even after 30 h incubation. Under in vitro conditions, the transformation of the trypanosomes into midgut forms was complete after 20–22 h incubation. Furthermore, the resulting parasites were morphologically indistinguishable from those that transformed in vivo. Similarly, the parasites transformed in vitro and in vivo could not infect mice.

Temperature was found to be an important factor in the transformation process. There was no transformation at 4° C, but the process was gradually stimulated by raising the temperature to 27° C. On the other hand, heating at 60° C appeared to have an irreversible effect on the ability of the midgut homogenates to transform the parasites, suggesting that the factors involved in the process are heat-labile. Blood-meal digestion in haematophagous insects is mediated by a wide variety of enzymes. In *Glossina* species, at least six proteolytic enzymes are involved in this process (Gooding and Rolseth 1976; Cheeseman and Gooding 1985). Of these, trypsin has received the most attention (Gooding and Rolseth 1976). In the present study, a correlation was established between trypsin activity and the ability of the midgut homogenates to induce the transformation of bloodstream trypanosomes. All of the factors that affected enzyme activity also affected transformation. For example, the stimulation of trypsin activity in midgut homogenates obtained by increasing the temperature from 4° to 27° C led to a concomitant stimulation of transformation. Similarly, heating destroyed both trypsin and the transformation activities of the midgut homogenates. Another correlation was found between the inhibition of trypsin activity by protease inhibitors and transformation.

Treatment of cultured trypanosomes with commercial trypsin has been reported to stimulate the transformation process at 27° C in the presence of a rat astroglioma cell line as the feeder layer (Yabu and Takayanagi 1988). In our experiments, blood was found to be important not only in stimulating the release of midgut enzymes but also in promoting the transformation process. An important observation was that an optimal trypsin concentration was crucial for the efficiency of the transformation process. High concentrations of the enzyme resulted in complete lysis of the parasites. High levels of protease activity have also been reported to cause damage to *Plasmodium* spp. (Yeats and Steiger 1981). Interestingly, the isolated parasites exerted a concentration-dependent inhibitory effect on trypsin activity in vitro. The presence of the parasites caused a reduction in trypsin activity amounting to approx. 70% in the gently teased midgut homogenates as compared with 60% in completely homogenised midguts. In the latter case, all of the parasites were lysed after about 4 h incubation. The mechanism underlying this inhibition is not yet clear.

The results presented in this report clearly indicate the involvement of midgut trypsin in the initiation of differentiation of bloodstream trypanosomes into procyclics. Whether the removal of the surface coat by trypsin alone is a sufficient signal for the process to occur, as has previously been suggested (Yabu and Takayanagi 1988), or whether other factors within the midgut also come into play remains a matter of conjecture. In addition, the role of blood in the process remains unclear. Further work is needed to elucidate all of the mechanisms involved.

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