***Tritrichomonas foetus* and *Trichomonas vaginalis*: the pattern of inactivation of hydrogenase activity by oxygen and activities of catalase and ascorbate peroxidase**

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The concentration-dependence of the inhibition of whole-cell hydrogen formation by oxygen has been measured in the trichomonads *Trichomonas vaginalis* and *Tritrichomonas foetus*, and compared with the oxygen inhibition of the *in situ* hydrogenase activity as measured by a tritium exchange assay. The inhibition profiles closely paralleled each other, suggesting that hydrogenase is the primary site of inhibition of anaerobic fermentative metabolism. In addition the inhibition profile for isolated hydrogenosomes was measured and shown to be similar to that for whole organisms. Ascorbate peroxidase was shown to be present in both organisms whereas catalase was confirmed to be present only in *Tritr. foetus*. The kinetic parameters of both enzymes were measured and their respective roles in oxygen protection discussed.

Keywords: *Tritrichomonas foetus*, *Trichomonas vaginalis*, hydrogenase, catalase, ascorbate peroxidase

INTRODUCTION

Trichomonads are a group of flagellated protozoans which differ from most other eukaryotes in their energy metabolism. The nature and subcellular organization of their bioenergetic systems have similarities to those of certain prokaryotes. A unique feature of trichomonads is the presence of hydrogenosomes and the absence of mitochondria. Amongst this group, *Trichomonas vaginalis* and *Tritrichomonas foetus* are widely studied. *Trich. vaginalis* is a sexually transmitted human parasite, infecting the urinogenital tract of both men and women; *Tritr. foetus* is a sexually transmitted parasite in cattle.

Trichomonads are microaerophilic organisms and even traces of oxygen influence their fermentative capability. At least part of the reason for this stems from the fact that they form hydrogen as a fermentative electron sink and the formation of hydrogen has been reported to be oxygen-sensitive (Lloyd & Kristensen, 1985; Ellis *et al.*, 1992). The measured oxygen tension in the vagina is significant (the physiological oxygen concentration range to which *Trich. vaginalis* could be exposed is 15–56 μM ; Wagner & Levin, 1978) and parasites are likely to be exposed to this oxygen tension as they reside in the host's urinogenital tract. Although hydrogen evolution by whole cells is known to be very sensitive to oxygen it has

not been shown whether this is attributable to inhibition of the hydrogenase enzyme itself. For example, it was shown by Pow & Krasna (1979) in green algae that oxygen inhibited hydrogen formation by cells but that this occurred under conditions where the hydrogenase itself was still active; it was concluded that oxygen inhibited the supply of electrons to the hydrogenase via an electron carrier. It has been reported that within the trichomonad hydrogenosomes there is another oxygen-sensitive enzyme, pyruvate:ferredoxin oxidoreductase (Lindmark & Müller, 1973; Müller, 1990). Hence, the nature of the effect of oxygen on the hydrogenase itself has not been resolved. In this study we have used the tritium exchange assay, which is specific for the hydrogenase enzyme itself, to measure the effect of oxygen on the activity of hydrogenase directly and have compared this with the pattern of oxygen inhibition of hydrogen metabolism of whole cells. We have also determined whether hydrogen formation by isolated hydrogenosomes is as sensitive to oxygen as is the hydrogen formation by whole cells.

Oxygen-protection mechanisms in trichomonads are not well understood. As well as direct oxygen-scavenging activities such as those catalysed by NAD(P)H oxidases, they would presumably also include scavenging mechanisms for superoxide and hydrogen peroxide, toxic

products of the reduction of oxygen by a variety of cellular processes. Hydrogen peroxide could be generated by the action of superoxide dismutase, which is known to exist in trichomonads; superoxide and hydrogen peroxide could arise from reactions in immune cells in the host in addition to that generated in the parasite itself. In many organisms, catalase is involved in the removal of hydrogen peroxide; cells also use peroxidases, which are effective in removing peroxide at low concentrations (Halliwell & Gutteridge, 1989). *Tritr. foetus* has been shown to possess catalase activity (Ryley, 1955; Müller, 1973) but this enzyme has not been reported in *Trich. vaginalis* (Ryley, 1955; Ninomiya & Suzuoki-Ziro, 1952). Glutathione peroxidase is found in many organisms but is not likely to be present in trichomonads, at least in *Tritr. foetus*, because glutathione is not present in this organism (Brown *et al.*, 1993). Many micro-organisms, including *Trypanosoma cruzi* (Boveris *et al.*, 1980), contain ascorbate peroxidase as a hydrogen peroxide scavenger.

The aims of this work were to answer two unresolved questions concerning the susceptibility of trichomonads to oxygen: (1) is the hydrogenase itself inactivated by oxygen, and (2) are catalase and ascorbate peroxidase both present in *Tritr. foetus* and *Trich. vaginalis* and what are their kinetic characteristics?

METHODS

Parasites. The human parasite *Trichomonas vaginalis*, an Australian isolate (WAA 38; Dr A. Warton, School of Biological Sciences, Macquarie University, Sydney), and the cattle parasite *Tritrichomonas foetus* (strain KV1) were kindly provided by Dr M. R. Edwards, School of Biochemistry and Molecular Genetics, University of New South Wales, Australia. The parasites were grown at 37 °C in a modified tryptose/yeast extract/maltose (TYM) medium, as originally described by Diamond (1955), but maltose was replaced with 14 mM glucose. *Trich. vaginalis* was grown at pH 6.2 and *Tritr. foetus* at pH 7.0. Growth medium (45 ml) was inoculated with cells (0.5 ml at a cell density of $5\text{--}15 \times 10^6$ cells ml⁻¹) in 50 ml polypropylene screw-capped tubes. For bulk culture this was scaled up to 8 ml cells in 800 ml culture flasks. The viability of the organisms was examined microscopically. Growth curves were obtained using a haemocytometer after fixing the cells before counting with 1% (w/v) paraformaldehyde and 0.5% sucrose in PBS (0.145 M NaCl; 0.15 M sodium phosphate) at pH 7.2. Cells in the late-exponential phase (*Tritr. foetus*, 22–24 h; *Trich. vaginalis*, 46–48 h) containing $5\text{--}15 \times 10^6$ cells ml⁻¹ were harvested by centrifugation (1465 g, 10 min, 4 °C) in a Sorvall RC-2B centrifuge with a GSA rotor.

Preparation of the hydrogenosomal fraction. The harvested cells were washed by centrifugation as above twice in sucrose (250 mM) and once in homogenizing buffer containing sucrose (250 mM), KCl (20 mM), KH₂PO₄ (10 mM), MgCl₂ (5 mM) and Tris/HCl (20 mM) adjusted to pH 7.0. The washed cells were then resuspended in approximately 5% (v/v) of the homogenizing buffer in a Thomas size B glass homogenizer and homogenized with 40–50 strokes of the teflon pestle at 900 r.p.m. at 4 °C. The homogenate was then adjusted to a volume of 15 ml with the same buffer and centrifuged as above for 5 min. The supernatant was separated and kept on ice while unbroken cells were sedimented and subsequently homogenized under identical conditions. This homogenate was then com-

bined with the supernatant and kept on ice. Almost 95% breakage was achieved.

The resulting homogenate was transferred into 30 ml capacity screw-capped tubes and was subjected to differential centrifugation in a Sorvall RC-2B refrigerated centrifuge using an SS-34 rotor. The nuclear fraction was sedimented (755 g, 4 min, 4 °C) and removed. The supernatant was then centrifuged (12100 g, 10 min) and the sediment was washed once with sucrose (250 mM) by centrifugation under identical conditions. The sediment obtained during this centrifugation step was the large particle fraction enriched in hydrogenosomes; it constituted about 14% of the total protein content as shown by Lindmark & Müller (1973). This large particle fraction is referred to as the hydrogenosomal fraction. The activity of frozen hydrogenosomal fractions was investigated in comparison with that of freshly prepared hydrogenosomal fractions. A hydrogenosomal fraction that had been frozen for 48 h at -20 °C retained about 80% of its hydrogen formation activity. The purity of the hydrogenosomal fraction was assessed by determining the activity of the hydrogenosomal marker enzyme malate dehydrogenase (decarboxylating) (Lindmark *et al.*, 1975; Hrdy *et al.*, 1993).

Maintenance of anoxic conditions. Experiments used to determine hydrogen gas formation were carried out under anoxic conditions. All the solutions for homogenizing and washing cells were sparged with argon for 30 min and kept in air-tight containers. Homogenization was performed under argon. The incubation media in 8.8 ml vials (Pierce Chemical) were set up in a manifold system and flushed for 20 min with nitrogen which was passed through a hot copper column heated electrically to about 300 °C.

Measurement of hydrogen formation. Whole cells were incubated in PBS containing NaH₂PO₄ (30 mM), NaCl (74 mM), CaCl₂ (0.6 mM), KCl (1.6 mM), pH 7.0, and supplemented with glucose (50 mM) (Cerkasovova *et al.*, 1984). The hydrogenosomal incubation medium consisted of KCl (20 mM), KH₂PO₄ (10 mM), MgCl₂ (5 mM) and Tris/HCl (20 mM), pH 7.0, and was supplemented with freshly prepared pyruvate (10 mM), ADP (1 mM) and succinate (100 µM) (Cerkasovova *et al.*, 1984). Incubation medium (4 ml) as described above was transferred into the screw-capped 8.8 ml vials. Different concentrations of oxygen were injected into the vials using a 1 ml gas-tight syringe. This was followed by injecting 100 µl sample (either whole cells or hydrogenosomal fraction) using a Hamilton microsyringe preflushed with argon. The sample was kept anoxically on ice prior to use. The vials were then incubated at 37 °C with shaking at 100 oscillations min⁻¹ (Steinbüchel & Müller, 1986). Gas samples (0.2 ml) were taken from the vials using a 1 ml disposable syringe (26-G needles), flushed with argon prior to use. While removing samples from the vials the syringes were pumped up and down five times, removed after about 50 s, and inserted quickly into rubber stoppers. Samples were then analysed by gas chromatography (Daday *et al.*, 1977).

Tritium exchange assay. Hydrogenase (EC class 1.12) activity was measured as tritium exchange activity. Incubation medium (2.2 ml) was transferred into 8.8 ml vials as above and rendered oxygen-free as described above. A sample (100 µl) of cells (0.5×10^6 cells ml⁻¹) was injected into the vials. For the oxygen-sensitivity test different concentrations of oxygen were introduced into the vials with a syringe. Details of the assay procedure are given by Ewart & Smith (1989).

Catalase assay. Catalase (EC 1.11.1.6) activity was assayed in a Clark-type oxygen electrode (Rank Bros). The reaction

mixture contained phosphate buffer (50 mM), pH 7.0, hydrogen peroxide (33.5 mM) and sample (homogenate or hydrogenosomal fraction) in a total volume of 3 ml. The temperature was maintained at 25 °C. During the assay, a stream of argon gas was bubbled through the buffer solution in the chamber by means of a hypodermic needle connected to the gas pressure regulator until all oxygen in the chamber was displaced. When the recorder registered zero oxygen, the system was allowed to equilibrate for a few minutes before adding 100 µl hydrogen peroxide which was prepared in the same buffer and also bubbled with argon. The chamber was then rapidly stoppered to minimize diffusion of air. The samples which had been previously incubated with 1% (v/v) Triton X-100 at room temperature were then added to the chamber using a Hamilton microsyringe. Because the presence of NAD(P)H oxidases in the sample could reduce the apparent activity, the samples were first desalted prior to assaying to remove endogenous substrates. The desalting column was a Pharmacia NAP-10 column which was washed with the same phosphate buffer used for the enzyme assay. The evolution of oxygen was recorded as a measure of catalase activity (Rio *et al.*, 1977; Nakano & Asada, 1981).

Ascorbate peroxidase assay. Ascorbate peroxidase (EC 1.11.1.11) was assayed spectrophotometrically using a Varian Techtron 635 spectrophotometer at 290 nm and 25 °C. The reaction mixture contained potassium phosphate (50 mM), pH 7.0, EDTA (0.1 mM), ascorbate (0.5 mM) and hydrogen peroxide (0.1 mM) in a total volume of 3.0 ml. The reaction was started by adding the sample and the decrease in the absorbance was recorded 10–30 s after this addition (Nakano & Asada, 1981). The samples were first desalted on a Pharmacia NAP-10 column.

Protein determination. Protein was determined by the Lowry method, adapted for microtitre plates. BSA was used as a standard.

Materials. Foetal calf serum was purchased from Cytosystems; tritium gas was obtained from Amersham; NAD and NADP were obtained from Boehringer Mannheim; hydrogen peroxide was obtained from BDM Chemicals; ADP and succinate were obtained from Sigma. All other chemicals were of analytical grade.

RESULTS

Investigation of the effect of oxygen on hydrogen formation and hydrogenase activity

A range of concentrations of oxygen was chosen to cover those of the physiological environment within the host. The uninfected human host's vaginal oxygen concentration has been reported to be 15–56 µM (Wagner & Levin, 1978) but the vaginal oxygen concentrations of infected humans have not been reported; neither have the oxygen concentrations of the reproductive tract of cattle, infected or uninfected. In order to obtain a full inhibition profile, the oxygen concentration was extended well beyond this range. The profile of oxygen inhibition of whole-cell hydrogen formation is shown in Fig. 1.

The tritium exchange reaction involves a phase transfer step, the substrate being a gas and the product being in the liquid phase. It was therefore necessary to choose assay conditions in which this phase transfer step was not rate-limiting. Hence the rate of tritium exchange was measured as a function of both time and cell concentration. The phase transfer was not rate-limiting up to a cell con-

Fig. 1. Effect of oxygen on the rate of hydrogen formation by whole cells (○) and hydrogenosomes (+) of *Tritr. foetus*, and the rate of tritium exchange activity of whole cells of *Tritr. foetus* (△) and *Trich. vaginalis* (×). The mean anoxic rates of hydrogen formation by whole cells and hydrogenosomes of *Tritr. foetus* were $0.84 \mu\text{mol h}^{-1} (\text{mg protein})^{-1}$ and $1.9 \mu\text{mol h}^{-1} (\text{mg protein})^{-1}$, respectively. The anoxic rates of tritium exchange activity of whole cells of *Tritr. foetus* and *Trich. vaginalis* were 973 c.p.m. and 713 c.p.m., respectively, both with a cell density of $0.5 \times 10^6 \text{ cells ml}^{-1}$.

centration of approximately $2 \times 10^6 \text{ cells ml}^{-1}$ but became so above this concentration; i.e. the rate of tritium exchange became zero-order with respect to enzyme concentration. Hence a cell concentration of $5 \times 10^5 \text{ cells ml}^{-1}$ was used in subsequent experiments. In these experiments it was not necessary to determine the degree of cellular isotopic quenching because the same number of cells was used at each oxygen concentration and therefore the relative magnitude of oxygen inactivation would not be artefactually affected by quenching. The oxygen inhibition profile for inhibition of tritium exchange activity is also shown in Fig. 1. It is clear that the profiles for inhibition of hydrogen production by whole cells and inhibition of tritium exchange activity are similar, indicating that inhibition of hydrogenase itself is the most probable cause of the inhibition of whole-cell hydrogen production.

The profile of oxygen inhibition of hydrogenosomal hydrogen formation

The pattern of hydrogenosomal hydrogen formation is also shown in Fig. 1. This was measured in order to investigate whether there are oxygen protection mechanisms in the cytoplasm which protect the hydrogenosomes. The fact that the profiles for each virtually coincide indicates that this is not the case.

Activities of catalase and ascorbate peroxidase

Catalase activity was confirmed to be present in *Tritr. foetus*, but not *Trich. vaginalis*. Its specific activity was $21 \pm 3 \mu\text{mol min}^{-1} (\text{mg protein})^{-1}$ (mean $\pm$ SD, $n = 5$). Ascorbate peroxidase activity was present in both *Tritr. foetus* and *Trich. vaginalis* [specific activities $0.43 \pm 0.05 \mu\text{mol min}^{-1} (\text{mg protein})^{-1}$ (mean $\pm$ SD, $n = 5$) and $0.36 \pm 0.03 \mu\text{mol min}^{-1} (\text{mg protein})^{-1}$ (mean $\pm$ SD, $n = 5$), respectively], with approximately 18% of the total activity present in the hydrogenosomal fraction. Kinetic analysis of the two enzymes revealed a hyperbolic function with respect to substrate concentration. The apparent K_m values for catalase and ascorbate peroxidase in *Tritr. foetus* were 55 ± 4 (mean $\pm$ SD, $n = 5$) mM and 35 ± 9 (mean $\pm$ SD, $n = 5$) μM , respectively. The K_m of the *Trich. vaginalis* ascorbate peroxidase was 49 ± 13 (mean $\pm$ SD, $n = 5$) μM .

DISCUSSION

The physiological oxygen concentration range to which *Tritr. foetus* could be exposed is 15–56 μM (Wagner & Levin, 1978). Over this oxygen concentration range the rate of hydrogen evolution by whole cells was substantially less than the anoxic rate (Fig. 1), the 50% inhibition concentration being about 25 μM . The pattern of oxygen inhibition of the hydrogenase itself was not distinguishable, within experimental error, from that on whole-cell hydrogen formation. This is consistent with the hydrogenase being a primary site of oxygen inhibition of anaerobic metabolism and supports the results of Ellis *et al.* (1992), who showed in *Trich. vaginalis* that hydrogenase activity was inhibited to a greater extent than pyruvate:ferredoxin oxidoreductase activity at 20 μM oxygen. Almost complete inhibition of hydrogenase activity was observed at ambient oxygen concentrations (195 μM). A similar pattern of inhibition was found for the hydrogenase in *Trich. vaginalis* (Fig. 1).

As shown in Fig. 1, within experimental accuracy the pattern of oxygen inhibition of hydrogen formation was similar in whole cells and in isolated hydrogenosomes, implying that whole cells offer no protection from oxygen over that available to the hydrogenosomes, in which the hydrogenase is located. Although the cytoplasm of the cells is known to contain NADH and NADPH oxidases (Müller, 1990), their primary function may be to dispose of surplus electrons generated by fermentative metabolism rather than to protect against oxygen.

In this work we also investigated the enzymes of hydrogen peroxide removal. Catalase activity was not detected in *Trich. vaginalis*, confirming what has been previously reported (Müller, 1990). We have also confirmed its presence in *Tritr. foetus* and have shown that both organisms contain ascorbate peroxidase. In *Tritr. foetus* the K_m value of catalase for hydrogen peroxide was much greater (55 mM) than that of ascorbate peroxidase (35 μM). The K_m of catalase from other sources varies from 47 to 1100 mM (Miyake *et al.*, 1991). Catalase is the more efficient enzyme at a higher hydrogen peroxide concentration [a specific activity of about $21 \mu\text{mol min}^{-1}$

(mg protein) $^{-1}$ compared with about $0.43 \mu\text{mol min}^{-1}$ (mg protein) $^{-1}$ for ascorbate peroxidase], whereas at low hydrogen peroxide concentrations ascorbate peroxidase would take over as the relatively more effective enzyme. Hence, as in many micro-organisms (Nakano & Asada, 1981; Miyake *et al.*, 1991), catalase is likely to play the role of efficiently removing most of any hydrogen peroxide that accumulates, with ascorbate peroxidase scavenging low concentrations of residual hydrogen peroxide. The kinetic properties of this enzyme in both *Tritr. foetus* and *Trich. vaginalis* were similar. It is not known why *Trich. vaginalis* does not have catalase. Perhaps *Trich. vaginalis* does not encounter the same levels of hydrogen peroxide *in vivo* as does *Tritr. foetus* but there is no evidence for this to our knowledge.

The question of whether hydrogenase is the primary site of inhibition of hydrogen formation is pertinent to other organisms which produce hydrogen. It appears that the situation in the trichomonads is quite different from that in green algae, for example, where oxygen was shown to inhibit whole-cell hydrogen formation under conditions in which the hydrogenase itself was still active (Pow & Krasna, 1979). This is not necessarily surprising, since in the algae hydrogen production is driven by electron flow through the photosynthetic membranes, whereas in the trichomonads hydrogen production is fermentative. It is also well known that hydrogenases from different sources vary markedly in their sensitivity to oxygen, some being totally irreversibly inhibited, others reversibly inhibited and yet others relatively unaffected (Adams *et al.*, 1981).

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