Hydrogen peroxide is a product of oxygen consumption by *Trichomonas vaginalis*

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The amitochondriate sexually-transmitted human parasitic protozoan *Trichomonas vaginalis* (Bushby strain) grown anaerobically on complex medium containing cysteine and ascorbic acid consumed O_2 avidly (6·9 μM min^{-1} per 10^6 organisms) with an apparent K_m value of 5·1 μM O_2 : O_2 uptake was inhibited by $O_2 > 120$ μM. Spectrophotometric assays in the presence of microperoxidase (419–407 nm) indicated that H_2O_2 was produced and that inhibition by high O_2 concentrations was again evident. Hydrogenosomes oxidizing pyruvate in the presence of ADP and succinate showed similar patterns of O_2 consumption, H_2O_2 production (33·5 pmol min^{-1} per mg protein), and O_2 inhibition. Cytosolic NADH oxidase gave no detectable H_2O_2, whereas the cytosolic NADPH oxidase produced H_2O_2 at a rate (43 pmol min^{-1} per mg protein) greater than that of hydrogenosomes. These results are discussed in relation to the oxidative stress experienced by the pathogen in its natural habitat.

1. Introduction

An understanding of the oxidative metabolism of the amitochondriate flagellated parasitic protozoan, *Trichomonas vaginalis*, an extremely common cause of human genito–urinary infections, is paramount in devising strategies for chemotherapy (Milne *et al* 1978; Lloyd and Petersen 1985). The redox-active organelles of trichomonads, the hydrogenosomes (Lindmark and Müller 1973) are responsible for the oxidation of pyruvate (Cerkasov *et al* 1978). Under anaerobic conditions hydrogenase is active and protons serve as the terminal electron acceptor for a chain of flavin and iron sulphur carriers (Lindmark and Müller 1973; Chapman *et al* 1986). When exposed to low O_2 concentrations (e.g., 20 μM O_2) the carbon fluxes through the alternative branching pathways from pyruvate are adjusted (Ellis *et al* 1992), hydrogenase activity becomes inhibited (Lloyd and Kristensen 1985), and O_2 serves as terminal electron acceptor (Lloyd *et al* 1983). NADH and NADPH oxidation also occurs in hydrogenosome-enriched fractions (Lindmark and Müller 1973; Lloyd *et al* 1983) with a variety of electron acceptors including O_2. *T. vaginalis* also possesses a highly active cytosolic NADH oxidase (Tanabe 1979; Brown *et al* 1996) and an NADPH: acceptor oxidoreductase (EC 1.6.99.1) which can use O_2 as electron acceptor (Linstead 1983; Linstead and Bradley 1987). As the vagina is not a strictly anaerobic habitat, but rather may have regions where O_2 can occasionally reach almost 60 μM (Wagner and Levin 1978), it is important to investigate O_2 scavenging as a mechanism of protection against O_2 toxicity (Ellis *et al* 1994) in this microaerophilic protozoan (Paget and Lloyd 1990). In this report we have determined the oxygen affinities of the major oxidases in the Bushby strain, and shown that H_2O_2 is a minor product, both in the hydrogenosomal and cytosolic compartments.

2. Methods

2.1 *Growth of the organisms and preparation of subcellular fractions*

T. vaginalis (Bushby strain) was grown with a logarithmic phase doubling time of 3·6 h in modified Diamond's medium as described previously (Chapman *et al* 1986). Disruption was with a motor-driven Potter–Elvejhem

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homogenizer with a Teflon pestle rotating at about 800 rev min^{-1} in a buffer containing 1 mM EDTA (Cerkasov *et al* 1978). The hydrogenosomal-enriched particulate fraction was obtained by centrifugation at 2·500 g_{av} for 15 min. The cytosolic fraction was prepared by centrifuging cell-free extracts at 94,000 g_{av} for 1 h.

2.2 *Dependence of O_2 uptake on O_2 concentration*

This was determined in the specially-constructed open O_2 electrode system (Lloyd and Kristensen 1985; Yarlett *et al* 1986) with windows for simultaneous spectrophotometric measurements. Argon (< 3 ppm O_2) was used to obtain anaerobiosis of the liquid in the vessel. One hundred per cent O_2 air and a standard gas mixture of 5% O_2 in N_2 was also used (Air Products, Cardiff, UK). Various O_2 tensions were obtained by the use of a digital gas mixer to provide O_2 dilutions in 5% steps. Apparent K_m O_2 values were obtained at low O_2 concentrations (0–50 μM). The evolving gas mixture (150 ml/min) was humidified by passage through water-soaked cotton wool before passage over the liquid vortex in the vessel. O_2 concentrations were calculated from percentage dilutions of stock gas mixtures.

Calibration for dissolved O_2 and determination of $t_{1/2}$ (half times for equilibration) were obtained using standard gas mixtures in the absence of biological material. Values for the O_2 concentration in the liquid phase (T_L) in the presence of respiring cells or subcellular fractions at a series of fixed values of O_2 concentration in the gas phase (T_G) then allowed calculation of respiration rates (from $V_r = k(T_G - T_L)$ where V_r = respiration rate and $k = \log_e 2/t_{1/2}$).

2.3 *Production of H_2O_2 by T. vaginalis cells and subcellular fractions*

Microperoxidase (MPII, Sigma) was used (3·0 μg/ml final concn.) for the detection of H_2O_2. The absorbance maxima of the free enzyme and the peroxide complex are at 407 nm and 419 nm respectively. The rate of H_2O_2 production was determined by the rate of change of absorption at 419–407 nm in the dual wavelength mode (molar absorption coefficient 78000 M^{-1} cm^{-1}).

3. Results

Figure 1a shows a double reciprocal (Lineweaver–Burk) plot of the glucose-supported dependence of O_2 consumption rates on O_2 tension by intact *T. vaginalis* cells. In a series of experiments at low O_2 concentrations (0–50 μM), the apparent K_m value was determined to be 5·12 ± 1·35 μM (SD from 3 experiments). The respiration of whole cells was inhibited by high oxygen concentrations (> 120 μM O_2). The extrapolated value for V_{max} of O_2 consumption was found to be 6·9 μM O_2/min/10^6 cells. H_2O_2 production by intact cells revealed a similar pattern of inhibition by high O_2 concentrations in the liquid phase (figure 1b). Thus both O_2 consumption rate and rate of H_2O_2 production increased as the O_2 concentration in solution was raised to a value of about 150 μM, above which, the cellular O_2 uptake and rate of H_2O_2 production decreased. The ratio of H_2O_2 produced to O_2 consumed increased as the O_2 concentration increased up to 250 μM (figure 1c).

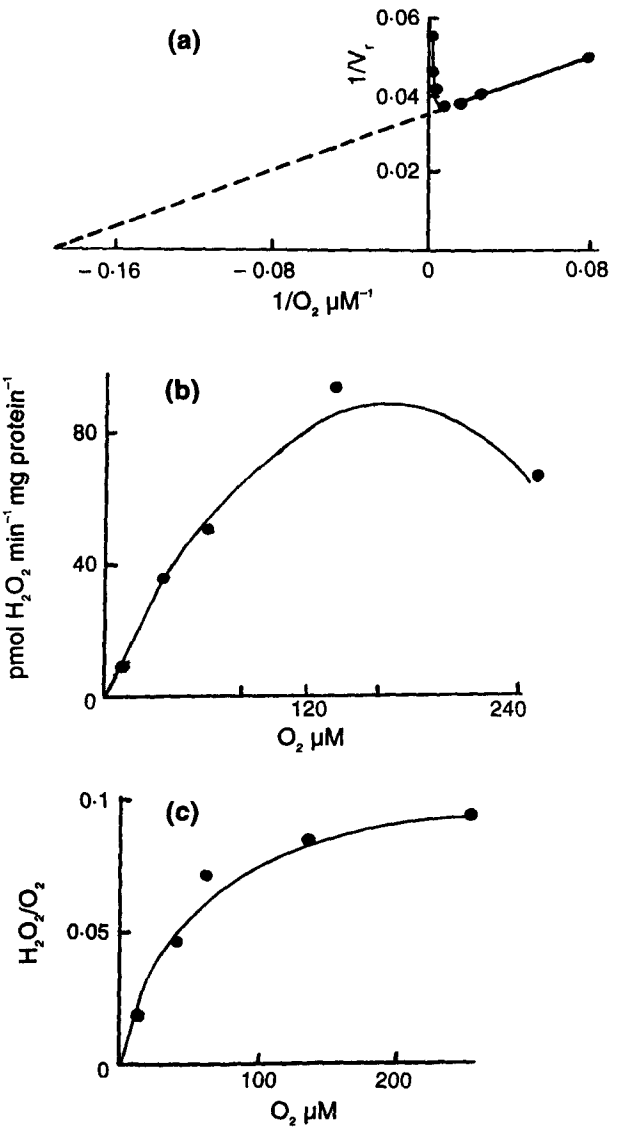

Figure 1. Dependence of O_2 consumption and H_2O_2 production rates on dissolved O_2 in washed cell suspension of *T. vaginalis* (Bushby strain). (a) Double-reciprocal plot for O_2 consumption. (b) H_2O_2 production. (c) Ratio of H_2O_2 produced to O_2 consumed at increasing O_2 concentrations. Intact cells (4×10^6 organisms/ml) were suspended in 150 mM potassium phosphate buffer pH 7·4. Glucose was added (final concn. 25 mM) and the liquid was stirred at 700 rev min^{-1}. The temperature of incubation was 37°C.

A similar pattern of O_2 uptake, H_2O_2 production, and inhibition was observed for hydrogenosomes oxidizing pyruvate in the presence of ADP and succinate, as for intact cells. Figure 2a shows the Lineweaver–Burk plot of hydrogenosomal O_2 consumption at low O_2 concentrations with an apparent K_m for O_2 of 11·2 μM. A value of 11·0 ± 3·2 μM (SD from 3 experiments) was obtained in a series of experiments. Inhibition of hydrogenosomal O_2 consumption occurred at an oxygen concentration of 110 μM, with an extrapolated V_{max} of 79·6 μM O_2/min/mg protein. The rate of production of H_2O_2 (figure 2b) increased up to an O_2 tension of 30 μM to a rate of 33·5 pmol/min/mg protein, above this O_2-concentration, production rapidly fell, to a rate of 11 pmol/min/mg protein. At 30 μM O_2, most of the O_2 consumed was converted to H_2O_2, but at high O_2 concentrations, the ratio of H_2O_2 production to O_2 consumption progressively decreased. Almost 14·4% of the O_2 consumed was converted to H_2O_2 at an O_2 concentration of 150 μM compared with 65% at a O_2 concentration of 30 μM (figure 2c).

The double reciprocal plot obtained for O_2 consumption rate against O_2 concentration by cytosolic NADPH oxidase (NADPH: flavin oxidoreductase) is shown in figure 3a. The apparent K_m for O_2 was determined after a series of experiments to be 27·2 ± 5·1 μM (SD from 3 experiments). The V_{max} was determined to be 33·1 μM O_2/min/mg protein. Cytosolic NADPH oxidase was shown to produce H_2O_2 (figure 3b). The rate of H_2O_2 produced (43·5 pmol/min/mg protein) was higher for the NADPH oxidase system than the hydrogenosomal O_2 uptake system. This production was decreased at an O_2 concentration greater than 90 μM: the ratio of H_2O_2 production to O_2 consumption at low O_2 concentrations was twice that of whole cells (figure 3c).

No increase in the rate of H_2O_2 production was observed when superoxide dismutase (SOD) (20 EU/ml) was added to the reaction vessel containing intact cells or subcellular fractions. The addition of catalase (5 EU/ml) prevented any further H_2O_2 production; i.e., no increase in absorbance of the microperoxidase complex was observed at 419–407 nm after its addition.

No H_2O_2 could be detected when the cytosolic fraction was incubated aerobically with NADH.

4. Discussion

The microaerophilic parasite, *T. vaginalis* (Paget and Lloyd 1990) is finely tuned so as to be able to utilize the very low (< 0·25 μM) levels of dissolved O_2 that accelerate hydrogenosomal acetate production (Ellis *et al* 1992) and hence substrate-level phosphorylation.

Presumably low levels of O_2 occur in microenvironments at the mucosal surface of the vagina. Increased blood flow can lead to more oxygenated conditions (Wagner and Levin 1978) and there may be other situations where elevated ambient O_2 can increase oxidative stress in the protozoon. We have previously shown that inhibition of O_2 consumption occurs when a threshold of O_2 concentration is exceeded, and that the critical level of dissolved O_2 varies from one strain of *T. vaginalis* to another (Yarlett *et al* 1986; Ellis *et al* 1994). One of these studies (Ellis *et al* 1994) confirmed earlier reports of the presence of superoxide dismutase activity in this (Linstead and Bradley 1988) and other anaerobic flagellates (Lindmark and Müller 1974). Other O_2-defense mechanisms include high levels of intracellular thiols especially methanethiol, propanethiol and H_2S (Ellis *et al* 1994). Therefore the sensitivity of *T. vaginalis* to O_2 at above physiological levels comes about from the production of H_2O_2, with the consequent likelihood of the generation of other reactive oxygen species in an organism where no catalase can be detected (Honigberg 1978).

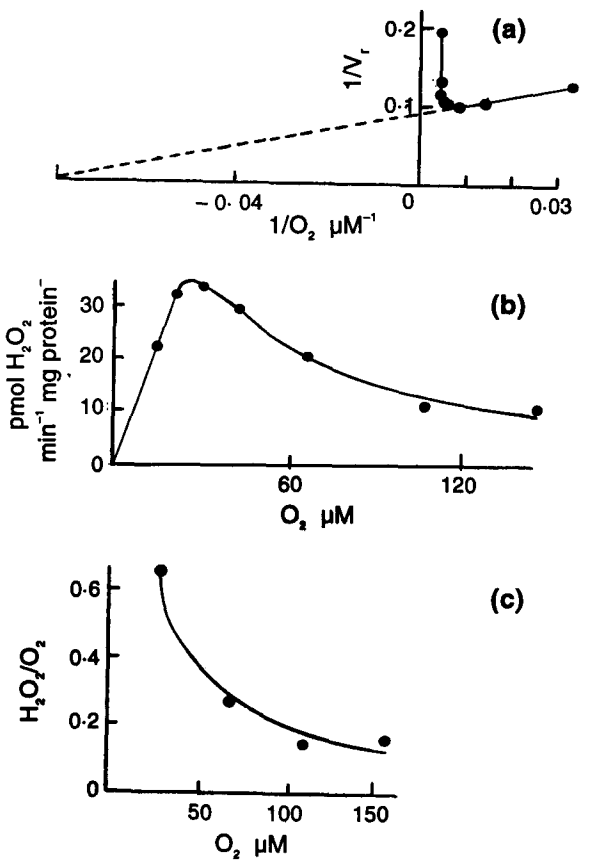

Figure 2. Dependence of O_2 consumption and H_2O_2 production rates on dissolved O_2 in a hydrogenosome-enriched fraction prepared from *T. vaginalis*. (a) Double-reciprocal plot for O_2 consumption. (b) H_2O_2 production. (c) Ratio of H_2O_2 produced to O_2 consumed at increasing O_2 concentrations. Hydrogenosome-enriched fraction (0·17 mg protein/ml final concn.) was resuspended at pH 7·1 (Cerkasov *et al* 1978). The respiratory substrates were (final concn.) pyruvate (40 mM), ADP (10 mM) and succinate (3 mM). Other conditions were as figure 1.

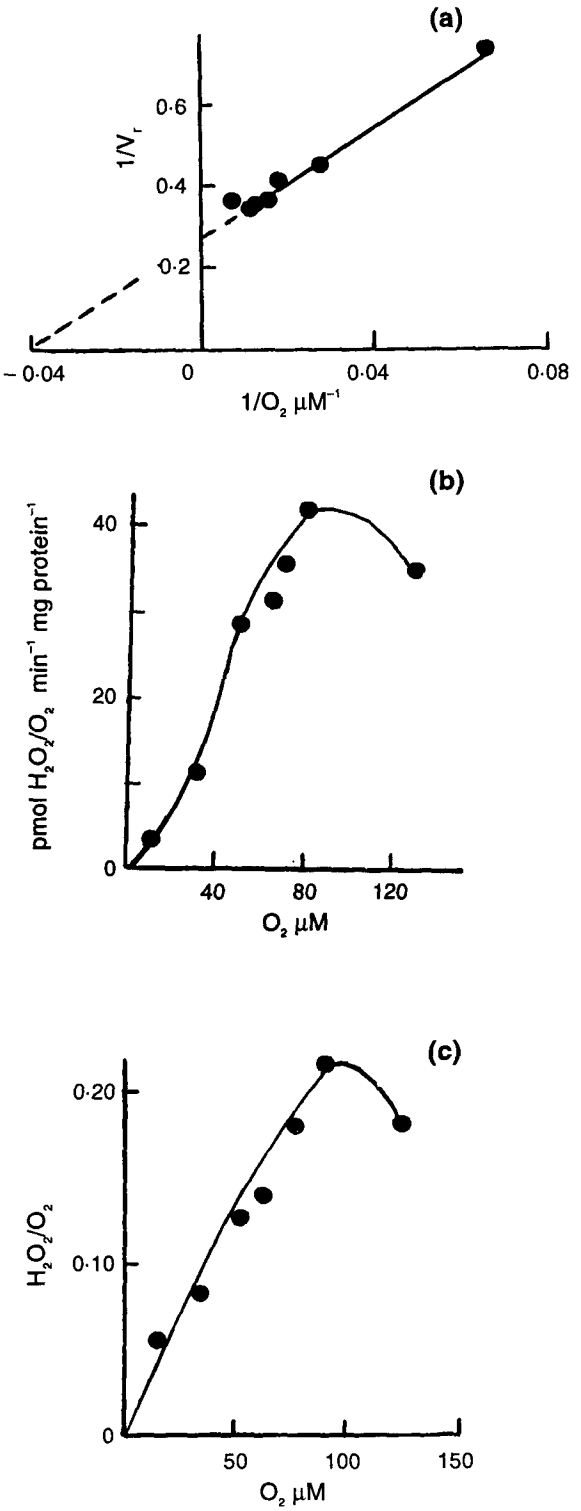

Figure 3. Dependence of O_2 consumption and H_2O_2 production rates of dissolved O_2 of cytosolic NADPH from *T. vaginalis*. (a) Double-reciprocal plot for O_2 consumption. (b) H_2O_2 production. (c) Ratio of H_2O_2 produced to O_2 consumed at increasing O_2 concentrations. The cytosolic fraction (0·31 mg protein/ml final concn.) was diluted in 150 mM potassium phosphate buffer, pH 7·4 to which was added NADPH (final concn. 0·2 mM). A concentrated solution of NADPH was made up in order to minimize the subsequent added volume (10 μl). NADPH was added periodically to ensure that respiration was not limited by the substrate concentration. Other conditions as figure 1.

The present work shows correlation of O_2 inhibition of O_2 uptake with H_2O_2 production both at the whole cell level as well as in cytosolic and hydrogenosomal-enriched fractions. Similar dependencies of both O_2 consumption and H_2O_2 generation rates suggests that this product of univalent reduction of the terminal electron acceptor arises at the cytosolic NADPH oxidase as well as at hydrogenosomal oxidases responsible for accepting electrons from pyruvate and NADPH.

The apparent K_m for O_2 of an organism is an indication of its ability to scavenge oxygen from its surroundings both for useful and protective functions. Its measured (apparent) value in intact cells depends upon (i) the nature of the terminal oxidase(s) (especially its affinity for O_2), and (ii) in large cells, the existence of O_2 gradients between the plasma membrane and sites of O_2 reduction. The ability of intact cells of *T. vaginalis* to remove O_2 from their surroundings is similar to that determined for the cattle parasite *Tritrichomonas foetus* KV1. Similar apparent K_m values for O_2 have been obtained for the hydrogenosome-containing anaerobic rumen holotrich *Dasytricha ruminantium*, and also for the free-living mitochondriate protozoan, *Tetrahymena pyriformis* (Lloyd *et al* 1982).

The apparent K_m value for O_2 obtained for the isolated hydrogenosomal or cytosolic fractions indicate that these subcellular preparations do not have such high affinities for O_2 as those exhibited by whole cells. The higher values of K_m for O_2 of both fractions may reflect either (i) the impairment of the activities of the fractions during processing and prolonged exposure to O_2 during the experiments, or (ii) loss of normal interaction between the hydrogenosome and the cytoplasm. The V_{max} values for O_2 consumption by hydrogenosomal or cytoplasmic NADPH oxidase obtained in the open system do not agree exactly with the values obtained from the rates of O_2 uptake recorded immediately after subcellular fractionation using the closed oxygen electrode. This is possible due to the extended times (about 2 h) in which the subcellular fractions are subjected to varying O_2 tensions in the open system; this exposure to O_2 may partially inactivate the enzyme system(s) acting as terminal oxidases.

From the results presented here, it is not possible to distinguish between reduced flavin (Massey *et al* 1971), an iron–sulphur centre (Misra and Fridovitch 1971), or superoxide as the source of hydrogenosomal or cytoplasmic H_2O_2 production. The decrease in the rate of H_2O_2 production observed at high O_2 concentrations can have several alternative explanations: (i) at high O_2 concentrations, the terminal oxidase is inhibited; this results in decreased H_2O_2 production; (ii) H_2O_2 itself becomes inhibitory to the terminal oxidase at high concentrations, i.e., product inhibition of the oxygen-utilizing systems of either the oxidase itself or an intermediate redox carrier by H_2O_2 may occur;

(iii) oxidative damage to the electron transport systems occurs as a result of H_2O_2 production. The primary reactant responsible for damage to the terminal oxidase systems may thus be oxygen itself, a product of its partial reduction such as H_2O_2, or a free radical such as superoxide or hydroxyl species. Because *T. vaginalis* contains no catalase, partial reduction of oxygen must result in a build-up of H_2O_2 capable of leading to irrepairable damage to sensitive cellular components including redox components of the electron transport chain.

Inhibition of H_2O_2 production by intact cells, hydrogenosomes and NADPH oxidase occurred at different oxygen concentrations. H_2O_2 production in hydrogenosomes was inhibited at a lower oxygen concentration than the overall oxygen consumption; this could indicate that H_2O_2 is not only produced by the terminal oxidase but also by another electron transfer component in the hydrogenosome. If superoxide radicals are produced by reduction of oxygen either at the terminal oxidase or elsewhere in the respiratory chain, then exogenous SOD added to the respiring system might not produce any effect if endogenous SOD activities are high. Indeed, addition of SOD to the reaction vessel did not increase the rate of change of absorbance when measuring H_2O_2 production by formation of the microperoxidase-H_2O_2 complex using intact cells or with either of the subcellular O_2 consuming systems.

Production of H_2O_2 by rat liver or pigeon heart mitochondria accounts for 0·3–2% of oxygen uptake under energetically-coupled or uncoupled physiological conditions respectively (Chance *et al* 1979). In mitochondria from a nematode worm energization also decreases H_2O_2 production (Paget *et al* 1987). Neither of the two O_2 consuming systems of *T. vaginalis* studied here produce H_2O_2 as the sole produce of O_2 reduction; the NADH oxidase in this organism produces only H_2O (Tanabe 1979; Brown *et al* 1996). At elevated oxygen tensions, the likelihood of H_2O_2 production increases. The production of H_2O_2 by *T. vaginalis* at low O_2 (< 20 μM) is probably of little significance in terms of toxicity, and presents no major problem to the organism. However, higher O_2 concentrations may evoke a potentially lethal situation as the organism becomes unable to excrete the H_2O_2 into its surroundings faster than it is produced. The resulting build-up of intracellular H_2O_2 produces irreversible cellular damage.

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