The Effects of Oxygen on Fermentation in *Tritrichomonas foetus* KV1 and its Variant 1MR-100 with Defective Hydrogenosomes

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The effects of low concentrations of O₂ on fermentation in the cattle parasite *Tritrichomonas foetus* KV1 and its variant 1MR-100 were compared using membrane inlet mass spectrometry to measure simultaneously and continuously ethanol, CO₂ and H₂. In strain KV1 glucose-supported H₂ and CO₂ production were stimulated by O₂ concentrations <1.4 µM but were inhibited at higher concentrations. Damped oscillatory responses in H₂ production indicated the operation of a feedback control system. Measurement of the O₂-dependence of O₂ consumption rates confirmed the presence of a high-affinity terminal oxidase (apparent $K_m = 1.6 \mu\text{M-O}_2$ at 37 °C) and substrate inhibition by O₂ at >8 µM-O₂. Successive periods of exposure to O₂ resulted in decreased O₂ scavenging capacity, as indicated by increasing apparent K_m values for O₂. The variant strain 1MR-100 which lacks pyruvate:ferredoxin oxidoreductase and hydrogenase showed quite different characteristics: H₂ production was not detectable, ethanol formation was inhibited by O₂ ($K_i = 1 \mu\text{M}$) and O₂-dependence of O₂ consumption indicated that no high-affinity oxidase was present (apparent $K_m = 33 \mu\text{M-O}_2$). Progressive increases in respiration rates on repeated exposure to low O₂ concentrations indicated a capacity for adaptation to aerobiosis.

INTRODUCTION

Trichomonads are flagellate protozoa, parasitic in animals and man (Honigberg, 1978*a, b*); they inhabit locales where periods of strictly anaerobic conditions may alternate with varying levels of O₂ supply (Wagner & Ottesen, 1982). The absence of mitochondria from these organisms (Lloyd *et al.*, 1979 *a, b*; Müller, 1980) necessitates the functioning of alternative oxidative sites; cytosolic systems and hydrogenosomes (Müller & Lindmark, 1978; Čerkasov *et al.*, 1978) both assume this role. In the cytosol highly active nicotinamide nucleotide oxidases specific for NADH (Tanabe, 1979) and NADPH (Linstead, 1983), are believed to play an important part in protecting the oxygen-sensitive enzymes of the hydrogenosome. The hydrogenosome itself has a complex chain of flavin and iron-sulphur electron carriers (Ohnishi *et al.*, 1980; Chapman *et al.*, 1986) using either protons or oxygen as terminal electron acceptors. The production of H₂ under anaerobic conditions is diminished in *Trichomonas vaginalis* when O₂ is admitted (Lloyd & Kristensen, 1985), and the organism has an intense respiration and shows a high affinity for O₂ (Lloyd *et al.*, 1982, 1987; Yarlett *et al.*, 1986).

In the cattle parasite *Tritrichomonas foetus*, succinate and glycerol (Chapman *et al.*, 1985) are produced in the cytosol, whereas acetate and molecular H₂ are produced in the hydrogenosomes (Müller, 1976). A metronidazole-resistant variant, *T. foetus* KV1-1MR-100 (Kulda *et al.*, 1984) lacks the hydrogenosomal enzymes pyruvate:ferredoxin oxidoreductase and hydrogenase (Čerkasovová *et al.*, 1984). Elimination of hydrogenosomal pyruvate metabolism in this strain leads to an increased glycolytic rate and the stoichiometric conversion of each molecule of glucose to two of ethanol plus two of CO₂.

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In the work described in this paper we used continuous monitoring of H_2 , O_2 , CO_2 and ethanol to compare the effects of low levels of O_2 on the fermentation products of *T. foetus* strain KV1 with those of strain KV1-1MR-100.

METHODS

Maintenance, growth and harvesting of the organism. *Tritrichomonas foetus* strains KV1 and KV1-1MR-100 were cultured in Diamond's liquid TYM medium without agar supplemented with 10% (v/v) heat-inactivated horse serum (Diamond, 1957). Stock cultures (5-5 ml in screw-capped tubes) were subcultured daily and used to inoculate 150 ml of culture medium in screw-capped bottles. Growth at 37 °C for 24 h gave a population of 1 to 2×10^6 organisms ml^{-1} . Organisms were harvested at room temperature in a bench centrifuge at 600 g_{av} for 2 min, and then washed twice in a phosphate-buffered salt solution, pH 6.9 (Doran, 1959).

Measurements of O_2 , CO_2 , H_2 and ethanol by mass spectrometry. A quadrupole mass spectrometer (Spectramass Dataquad; Spectrum Scientific, Congleton, Cheshire, UK) was used: the inlet consisted of a quartz tube (3.5 mm outside diameter) sealed at one end and having a 0.25 mm diameter hole 5 mm from its sealed end; silicone rubber sleeve (outside diameter 0.19 mm; inside diameter 0.15 mm) was stretched into place over the orifice. Measurements were made in a 2 ml polycarbonate tube (1 ml working vol.), with the mass spectrometer inlet submerged in reaction mixture. Gas mixtures were bubbled continuously through the cell suspensions at a constant flow rate (30 $ml\ min^{-1}$) via a nylon tube. The temperature was maintained at 37 °C by thermostatically heated water which was circulated around the reaction vessel. Gas mixing was done with a single two-way micro-solenoid valve (type 65.231; Kuhnke, Penn. Bucks, UK) with constant pressure gas inlets and microcomputer-regulated time-shared-switching between inlets; argon and 5% (v/v) O_2 in N_2 were the two gases used.

The vacuum system used for the mass spectrometer was a Pfeiffer Turbomolecular pump (type TPV050) backed by a two-stage Edwards rotary pump (E2M2).

The half-times for equilibration for O_2 , CO_2 , H_2 and ethanol in this system were 0.4, 0.3, 0.55 and 0.7 min respectively giving gas exchange coefficients of 1.73, 2.31, 1.26 and $1.0\ min^{-1}$. Gas solubilities were taken as being 220 μM for O_2 in air-saturated Doran's buffer, and 705 μM for saturated H_2 (Wilhelm *et al.*, 1977); CO_2 calibration was done by adding standard $NaHCO_3$ solutions to the reaction mixture at pH 6.9.

RESULTS AND DISCUSSION

Fig. 1 shows the effects of adding low concentrations of O_2 ($< 2\ \mu M$) on fermentation in a non-proliferating cell suspension of *T. foetus* KV1. Adding 10 mM-glucose gave 30% stimulation of H_2 and 20% stimulation of CO_2 production under anaerobic conditions; the rate of ethanol formation was so low as to be barely detectable ($< 0.05\ nmol\ min^{-1}$ per 10^6 cells). Production rates both of CO_2 and H_2 showed an overshoot followed by a damped oscillation on adding glucose. Mixing 0.25 kPa O_2 into the mobile gas phase resulted in a rapid equilibration to a steady state concentration of 0.32 μM - O_2 in solution. This low concentration of O_2 stimulated H_2 production (9%) and CO_2 production (17%). Further stimulation of H_2 production was observed on increasing dissolved O_2 to 0.53 μM ; at O_2 concentrations greater than 1.3 μM , the H_2 concentration began to decline. The concentration of CO_2 increased until O_2 exceeded 1.6 μM . Overshoots both in H_2 and CO_2 concentrations were evident after every O_2 addition.

Increasing O_2 concentrations further gave progressively decreased H_2 and CO_2 production (not shown); K_i values for the inhibition by O_2 were 2.3 and 2.5 μM respectively.

Oxygen consumption by strain KV1 (Fig. 2) showed a complex dependence on O_2 concentration which was modified by prolonged exposure to O_2 concentrations in excess of about 10 μM . Thus, at first, organisms maintained anaerobically showed a high affinity for O_2 (apparent K_m value 1.6 μM) and substrate inhibition above 8 μM - O_2 . Each O_2 concentration was maintained for 10 min giving a total exposure time of 1 h; then the mobile gas phase was switched back to argon and anaerobic conditions re-established. When the step increases of O_2 were repeated with the same batch of organisms the respiration rate was lower for any given value of dissolved O_2 and the affinity for O_2 as determined from a double reciprocal plot was decreased. Successive series of stepwise exposures to O_2 produced further increases in the apparent K_m value.

Plot 1(a) (Fig. 2) shows the O_2 -concentration-dependence of respiration at high cell density (3.3×10^7 organisms ml^{-1}) and gives the same extrapolated apparent K_m value (1.6 μM - O_2) as that obtained at half that cell density (plot 1, Fig. 2). On exposure to O_2 , washed non-

Fig. 1. Effects of low O_2 concentrations on ethanol, H_2 and CO_2 production by *T. foetus* KV1 during glucose utilization. Washed organisms ($2.7 \times 10^7 \text{ ml}^{-1}$) were suspended at 37°C in Doran's buffer (pH 6.9) under an atmosphere of argon. Additions were made or the composition of the mobile gas phase was altered at the points marked by arrows. (a) Ethanol ($m/z = 31$); (b) H_2 (.....), CO_2 (—) and O_2 (-----) measured at $m/z = 2, 44$ and 32 respectively.

Fig. 2. O_2 -dependence of glucose-supported O_2 consumption rates in *T. foetus* KV1. Plot 1: conditions and measurements as in Fig. 1 except that 1.5×10^7 organisms ml^{-1} were exposed to equal stepwise increases of O_2 (up to 2 kPa final pO_2); each concentration was maintained for 10 min and after the fifth step the gas phase was switched to argon. The process was repeated three times (plots 2–4). For plot 1 (a) 3.3×10^7 organisms ml^{-1} were exposed to stepwise O_2 increases. Apparent K_m values (μM) for O_2 are indicated in **bold type**.

Fig. 3. Effects of low O_2 concentrations on ethanol and CO_2 production by *T. foetus* KVI-1MR-100 during glucose utilization. Conditions as in Fig. 1 except that the reaction vessel contained 2.5×10^7 organisms ml^{-1} . (a) Ethanol (—) and H_2 (.....); (b) CO_2 (—) and O_2 (-----).

proliferating organisms progressively lost the capacity to produce H_2 , and their motility decreased. Whether recovery of these functions is possible if these organisms are returned to growth medium has not been assessed. The slow production of ethanol by this strain was not measurably increased during O_2 exposure.

For strain KVI-1MR-100 a very different pattern of responses to O_2 was obtained (Fig. 3). Under anaerobic conditions, organisms utilizing glucose showed a high rate of ethanol production and hydrogen was undetectable. As fermentation of glucose commenced a burst of ethanol formation was accompanied by an extensive overshoot in the concentration of dissolved CO_2 which relaxed to about a half its maximum value within 15 min. Oxygenation to $0.5 \mu M$ O_2 gave a marked reduction (about 30%) in the ethanol accumulation rate [$K_i = 1.0 \mu M \pm 0.04$ ($n = 5$)].

Under anaerobic conditions linear accumulation of ethanol was observed during fermentation of glucose over periods of up to 1 h and up to 2 mM-ethanol.

Further stepwise increases produced only slight stimulation of CO_2 production (much less so than was the case for strain KVI), and the O_2 -induced overshoots in CO_2 production observed with the 'normal' strain were not observed. Double reciprocal plots for the O_2 dependence of respiration rates of the variant strain indicated a linear relationship (Fig. 4): the maximum respiratory rates were increased and the apparent K_m value for O_2 was greatly increased by comparison with strain KVI. Exposure to O_2 gave an adaptation to O_2 utilization rather than

Fig. 4. O_2 dependence of glucose-supported O_2 consumption rates in *T. foetus* KV1-1MR-100. Conditions, measurements and gas mixing programme were as in Fig. 2 except that the suspension contained 6.3×10^6 organisms ml^{-1} . The apparent K_m value (μM) for O_2 is indicated in **bold type**.

Table 1. Characteristics of O_2 consumption rates of product formation by *T. foetus* KV1 and its variant KV1-1MR-100

Organisms were incubated in the reaction vessel of a mass spectrometer (see Methods) in the presence of 20 mM-glucose; results are means $\pm$ SD (number of determinations in brackets). ND, Not detectable i.e. <0.02 nmol min^{-1} per 10^6 cells.

	O_2 consumption		Anaerobic production rate (nmol min^{-1} per 10^6 cells) of:	
	V_{max} (nmol min^{-1} per 10^6 cells)	Apparent K_m (μM)	H_2	ethanol
KV1	3.4 ± 3.7 (6)	1.6 ± 0.4 (6)	0.55 ± 0.02 (2)	0.05 ± 0.01 (4)
KV1-1MR-100	6.3 ± 2.7 (12)	32.8 ± 14 (10)	ND	1.43 ± 0.18 (5)

loss of respiratory capacity as observed in strain KV1. A summary of kinetics of fermentation and O_2 consumption in the two strains (Table 1) indicates that loss of the hydrogen-producing capability in the variant strain is accompanied by reduced O_2 -scavenging capacity. The oxygen-sensitive enzymes, pyruvate:ferredoxin oxidoreductase and hydrogenase are not present in the variant strain (Čerkasovová *et al.*, 1984) and this may explain its increased O_2 tolerance.

Further work is necessary to characterize the high-affinity oxidase of hydrogenosomes and the mechanism whereby it is inactivated by O_2 . Cytochromes are absent from all the trichomonads studied, and it has been argued that a flavoprotein or an iron-sulphur protein are unlikely candidates in this role (Ohnishi *et al.*, 1980; Chapman *et al.*, 1986).

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