Effect of fixation on activity and cytochemistry of hydrogenosomal enzymes in *Trichomonas vaginalis*

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The effect of fixation on the activity of malate dehydrogenase (decarboxylating) and pyruvate synthase was investigated in *Trichomonas vaginalis*. Subsequently a cytochemical staining method was developed for the demonstration of malate dehydrogenase activity in hydrogenosomes. After fixation of cells in low concentrations of glutaraldehyde and incubation in the presence of malate and the tetrazolium compound 2-(2'-benzothiazolyl)-5-styryl-3-(4'-phthalhydrazidyl)tetrazolium chloride, an electron-dense deposit was produced in the hydrogenosomes. During the whole procedure strictly anaerobic conditions were required. Attempts to develop an analogous procedure for pyruvate synthase failed because even low concentrations of glutaraldehyde strongly inhibited enzyme activity. When cells were fixed in low concentrations of glycolaldehyde and acetaldehyde, a high enzyme activity was retained, but no staining could be achieved. Application of both staining methods to the sapropelic ciliates *Trimyema compressum* and *Plagiopyla nasuta* gave negative results.

Introduction

Trichomonad flagellates, rumen ciliates and rumen fungi are microaerotolerant anaerobic organisms that lack mitochondria but contain hydrogenosomes (Müller, 1988; Lloyd *et al.*, 1989; Yarlett *et al.*, 1986). These organisms have a fermentative metabolism. Carbohydrates are incompletely oxidized to organic end products and CO₂. Under anaerobic conditions H₂ is produced (Mack & Müller, 1980; Lindmark *et al.*, 1989; Borneman *et al.*, 1989). Hydrogenosomes play a key role in the metabolism of the anaerobic protozoa and are characterized by several marker enzymes e.g. hydrogenase, pyruvate synthase (pyruvate:ferredoxin 2-oxidoreductase; EC 1.2.7.1) and malate dehydrogenase (decarboxylating (EC 1.1.1.39). In a previous study, a cytochemical staining method for hydrogenase was described and used to demonstrate the hydrogenosomal nature of the microbody-like organelles of sapropelic ciliates (Zwart *et al.*, 1988). Together with a study on a *Trichomonas vaginalis* isolate (K. B. Zwart & P. C. Raemakers-Franken, unpublished results) it showed the great

influence of fixation on enzyme activity. The present paper describes the effect of fixing agents on the activity of malate dehydrogenase (decarboxylating) and pyruvate synthase in *Trich. vaginalis*. Furthermore, a method for the cytochemical localization of malate dehydrogenase (decarboxylating) in hydrogenosomes of *Trich. vaginalis* is presented.

Methods

Organisms and cultivation. *Trichomonas vaginalis* strain C-1:NIH (ATCC 30001) was a gift from Professor D. Lloyd (Cardiff, UK) and was cultivated at 37 °C in a tryptose/yeast extract/maltose medium (Diamond, 1957), pH 6.8, supplemented with 5% (v/v) heat-inactivated horse serum. Exponentially growing cells (15 ml, 10⁶ cells ml⁻¹) were harvested under anaerobic conditions by centrifugation at 1000 g for 10 min.

Trimyema compressum strain K was isolated from a polluted ditch in Konstanz, FRG, and cultivated monoxenically in CM1 medium at 28 °C with *Bacteroides* sp. strain WoCb15 as food bacteria (Wagener & Pfennig, 1987). For growth of the food bacteria the medium was supplemented with 5 mM-tartaric acid. *Trim. compressum* strain N was isolated from a sludge backing pond of a waste-water treatment plant near Nijmegen, The Netherlands (Goosen *et al.*, 1990a), and cultivated monoxenically as described for strain K. Exponentially growing cells (40 ml, 1–3 × 10³ cells ml⁻¹) of strain K and N were harvested under anaerobic conditions by centrifugation at 1000 g for 5 min.

Plagiopyla nasuta was isolated from a sapropel sample and cultured in an undefined mixture of bacteria (Goosen *et al.*, 1988). Cells were

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Abbreviation: BPST, 2-(2'-benzothiazolyl)-5-styryl-3-(4'-phthalhydrazidyl)tetrazolium chloride.

harvested anaerobically from exponentially growing cultures (4 ml, 100–200 cells ml⁻¹).

Enzyme activity of *Trich. vaginalis*. Cells were harvested and washed in 0.1 M-KH₂PO₄/K₂HPO₄ buffer (PPB), pH 7.0, containing 0.25 M-sucrose.

Malate dehydrogenase (decarboxylating) activity of cell homogenates was measured spectrophotometrically with NAD (0.33 mM) at 37 °C under anaerobic conditions according to the method of Lindmark & Müller (1974). Homogenates were prepared by disrupting the cells with 20 strokes in a Potter homogenizer fitted with a glass pestle. The effect of fixation on malate dehydrogenase (decarboxylating) activity was studied by treating the cells with different concentrations of formaldehyde and glutaraldehyde in PPB plus sucrose for 10 min at 0 °C under anaerobic conditions. Homogenates of fixed cells were prepared after three rinses in PPB plus sucrose.

Pyruvate synthase activity of intact cells was measured spectrophotometrically with methyl viologen as electron acceptor at 37 °C under a N₂ atmosphere (Lindmark & Müller, 1973). β -Mercaptoethanol was omitted since the reaction was measured under strictly anaerobic conditions. The effect of fixation on pyruvate synthase activity was studied by treating the cells with different concentrations of formaldehyde, glutaraldehyde, acetaldehyde and glycolaldehyde in PPB plus sucrose for 10 min at 0 °C under anaerobic conditions. Before measuring the enzyme activity, the cells were rinsed three times in PPB plus sucrose.

Protein was measured according to the method of Sedmak & Grossberg (1977) with bovine serum albumin as standard. When the effect of fixation on enzyme activity was studied, samples for protein measurement were taken before addition of the fixatives.

Cytochemical staining. Cells of *Trich. vaginalis* were harvested and rinsed in PPB plus sucrose. For cytochemical staining of malate dehydrogenase (decarboxylating) activity the cells were prefixed for 10 min at 0 °C in 0.5% (v/v) glutaraldehyde in PPB plus sucrose and rinsed again three times in the same buffer to remove the glutaraldehyde. Prefixed cells were incubated anaerobically in serum flasks (10 ml) for 60–100 min at 24 °C in PPB plus sucrose containing malate (20 mM) and 2-(2'-benzothiazolyl)-5-stryryl-3-(4'-phthalhydrazidyl)tetrazolium chloride (BSPT, 0.25 mg ml⁻¹) as electron acceptor (Zwart *et al.*, 1988; Doddema *et al.*, 1979; Shannon, 1982), with N₂ (130 kPa) in the headspace. In control experiments malate was omitted.

For cytochemical staining of pyruvate synthase activity the cells were prefixed for 10 min at 0 °C in a mixture of 1% (w/v) glycolaldehyde and 1% (v/v) acetaldehyde in PPB plus sucrose and rinsed three times in the same buffer. Prefixed cells were incubated at 24 °C as described above, except that malate was replaced by 20 mM-pyruvate. In control experiments pyruvate was omitted.

Both enzyme reactions were followed by light microscopy and stopped by rinsing the cells twice in 0.1 M-Tris/HCl, pH 7.0, containing 0.25 M-sucrose. Up to this point strictly anaerobic conditions were maintained. The cells were postfixed for 30 min at 0 °C in a mixture of 2% (v/v) glutaraldehyde and 1% (w/v) OsO₄ in 0.1 M-cacodylate buffer, pH 7.0. Reduced BSPT, formed during the incubations, was modified into an electron-dense product in a reaction with the OsO₄ (Shannon, 1982). After three rinses with distilled water the cells were stained with 1% (w/v) uranyl acetate in 70% (v/v) ethanol. Specimens were dehydrated in an ethanol series and embedded in Araldite or Epon 812. Ultrathin sections, cut with a diamond knife, were poststained with lead citrate (Reynolds, 1963) and examined with a Philips EM 201 microscope.

Sapropelic ciliates. Cytochemical procedures for sapropelic ciliates were applied as described for *Trich. vaginalis* except that the cells were prefixed immediately after harvesting.

All solutions were kept in serum flasks closed with butylrubber stoppers. Oxygen was eliminated by three cycles of evacuation and gassing with 130 kPa N₂. Gases were made oxygen-free by passage over a catalyst: prereduced BASF R3-11 at 150 °C. All procedures with opened flasks, for which anaerobic conditions were essential, were done in an anaerobic glovebox.

Results and Discussion

The effect of fixing agents on the activity of malate dehydrogenase (decarboxylating) and pyruvate synthase in *Trich. vaginalis* is given in Table 1.

Malate dehydrogenase (decarboxylating)

Unfixed whole cells did not show a measurable enzyme activity. This might be due to the existence of a permeability barrier for exogenous substrates (Müller, 1973). Homogenates of unfixed cells showed a specific activity of 130 nmol min⁻¹ (mg protein)⁻¹. Table 1 shows that the specific enzyme activity was strongly inhibited when cells were fixed in formaldehyde or glutaraldehyde at concentrations of 1% or more. At glutaraldehyde concentrations of 0.5% or less, 70–100% of the original enzyme activity was retained.

Table 1. Effect of fixation on the malate dehydrogenase (decarboxylating) and pyruvate synthase activity in *Trich. vaginalis*

The table gives the data from one experiment.

Fixing agent	Concn (%)	Relative specific activity (%)	
		Malate dehydrogenase*	Pyruvate synthase†
Formaldehyde	0.25		15
	0.5		0
	1	21	
	2	0	
Glutaraldehyde	0.25	70–100‡	21
	0.5	75–100‡	16
	1	50	0.6
	2	34	
	2.5	0	
Formaldehyde + glutaraldehyde	1 + 1	0	
Glycolaldehyde	0.25		107
	0.5		101–145‡
	1		246
	2		171
Acetaldehyde	0.25		50
	0.5		170

* Enzyme activity measured in homogenates. Specific activity (100%) in homogenates of unfixed cells was 130 nmol min⁻¹ (mg protein)⁻¹.

† Enzyme activity measured in whole cells. Specific activity (100%) of unfixed cells was 580 nmol min⁻¹ (mg protein)⁻¹.

‡ Data from two independent experiments.

Fig. 1. Light (*a*, *b*) and electron (*c*, *d*) micrographs of *Trich. vaginalis* fixed anaerobically in 0.5% glutaraldehyde and stained for malate dehydrogenase activity with BSPT as electron acceptor. (*a*) Cells incubated in the presence of malate showing positively stained granules. (Bright field.) Bar, 5 μ m. (*b*) Control cells incubated in the absence of malate showing no staining. (Bright field.) Bar, 5 μ m. (*c*) Cell incubated in the presence of malate. A positive reaction is shown in the hydrogenosomes. Bar, 1 μ m. (*d*) Control cell. No staining is visible. Bar, 1 μ m.

Pyruvate synthase

With unfixed cells of *Trich. vaginalis* a specific enzyme activity of $580 \text{ nmol min}^{-1} (\text{mg protein})^{-1}$ was measured. Table 1 shows that even at low concentrations of glutaraldehyde or formaldehyde the specific enzyme activity was strongly inhibited. Consequently these fixatives could not be used for cytochemical staining of pyruvate synthase. However, after fixation with low concentrations of glycolaldehyde or acetaldehyde, a high enzyme activity was retained. Obviously, the fixation procedures actually stimulated the measurable enzyme activity. This may be due to an increased permeability of the cells.

Cytochemical staining of *Trich. vaginalis*

When cells were fixed anaerobically in 0.5% glutaraldehyde and subsequently incubated in the reaction mixture for malate dehydrogenase (decarboxylating) staining,

blue-violet-stained granules were found inside the cells (Fig. 1*a*). In control incubations staining was absent (Fig. 1*b*). In ultrathin sections an electron-dense deposit was observed exclusively in the hydrogenosomes of the experimental cells (Fig. 1*c*). The whole matrix of the hydrogenosomes was stained, in contrast to cytochemical staining of hydrogenase, where the matrix of some hydrogenosomes remained unstained (Zwart *et al.*, 1988). No difference was observed between cells embedded in Epon and Araldite. In control cells no deposit was observed (Fig. 1*d*). The formation of the blue-violet-stained formazan could not be inhibited when the cells were rinsed in the presence of 1 mM-EDTA before and after fixation, and subsequently incubated in the presence of 1, 2 or 5 mM-EDTA. Although malate dehydrogenase (decarboxylating) activity can be measured under aerobic conditions (Müller, 1973; Lindmark & Müller, 1974), for cytochemical staining of this enzyme strictly anaerobic conditions were required until the reduction of BSPT was completed. The reason

for the oxygen sensitivity during the staining procedure is not known.

When, after the reaction, cells were rinsed with PPB plus sucrose instead of Tris/HCl plus sucrose, a non-specific precipitate was formed in both experimental and control cells (results not shown).

The fact that a positive reaction occurred only in the hydrogenosomes and not in the cytoplasm implies that only the decarboxylating malate dehydrogenase ('malic enzyme') is measured with the described method and not the cytoplasmic malate dehydrogenase (= oxaloacetate reductase), which is also present in *Trich. vaginalis* (Brugerolle & Méténier, 1973; Steinbüchel & Müller, 1986). The failure to demonstrate an inhibiting effect of EDTA on the cytochemistry of decarboxylating malate dehydrogenase (Lindmark *et al.*, 1975) may be explained by the inability of EDTA to permeate the cell and hydrogenosomal membranes. Furthermore, it is very unlikely that formazan will cross the hydrogenosomal membrane after it is formed during the reaction. Besides hydrogenase (Zwart *et al.*, 1988), malate dehydrogenase (decarboxylating) is the second hydrogenosomal enzyme demonstrated by *in situ* cytochemical localization.

Pyruvate synthase

Although the measurements show an increased activity of pyruvate synthase after fixation with 1% glycolaldehyde it was not possible to visualize the presence of this enzyme by cytochemical staining. Even after fixation in a mixture of 1% glycolaldehyde and 1% acetaldehyde, and with an increase of the incubation time to 20 h, no staining could be detected. A reason might be that the treatment with glycolaldehyde and acetaldehyde does not make the cells permeable to BSPT. After 3–4 h of incubation of experimental and control cells, only those cells which had lost their original shape and had become rounded contained blue-violet-stained granules. Another reason might be that pyruvate synthase will not reduce BSPT at all. Furthermore, this enzyme is extremely oxygen labile.

Sapropelic ciliates

After establishing the method for malate dehydrogenase (decarboxylating) localization in *Trich. vaginalis*, the same technique was applied for this enzyme in the anaerobic sapropelic ciliates *Trim. compressum* and *P. nasuta*. However, no positive results were obtained with these organisms. Even when the cells were incubated for 20 h, no difference could be observed between experimental and control cells. The failure to demonstrate the presence of malate dehydrogenase (decarboxylating) in the sapropelic ciliates may be explained by a low activity

of this enzyme. It is probably not due to the omission of Mn^{2+} because in an anaerobic, hydrogenosome-containing flagellate (Broers *et al.*, 1989) the assay showed negative results in both the absence and presence of Mn^{2+} . As has been demonstrated for some rumen ciliates (Yarlett *et al.*, 1981, 1984), this enzyme may even be totally absent in the hydrogenosomes. Although the presence of the cytoplasmic oxaloacetate reductase was shown in *Trim. compressum* strain K (Wagener, 1989), as in *Trich. vaginalis*, it was not possible to demonstrate this enzyme by the described staining method.

Also, trials to demonstrate pyruvate synthase in *Trim. compressum in situ* remained as unsuccessful as those for *Trich. vaginalis*.

Hydrogenase activity has been demonstrated in the microbodies of the sapropelic ciliates *Trim. compressum* and *P. nasuta*. This fact indicates that the microbodies present may be hydrogenosomes (Zwart *et al.*, 1988). Moreover, hydrogen production by *Trim. compressum* strain N has been demonstrated (Goosen *et al.*, 1990b), and the cells lack cytochromes and cytochrome oxidase (Goosen *et al.*, 1990a). The present results show that attempts to stain two more hydrogenosomal enzymes in the sapropelic ciliates remain unsuccessful.

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