

Iron-induced changes in pyruvate metabolism of *Tritrichomonas foetus* and involvement of iron in expression of hydrogenosomal proteins

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The main function of the hydrogenosome, a typical organelle of trichomonads, is to convert malate or pyruvate to H₂, CO₂ and acetate by a pathway associated with ATP synthesis. This pathway relies on activity of iron-sulfur proteins such as pyruvate:ferredoxin oxidoreductase (PFOR), hydrogenase and ferredoxin. To examine the effect of iron availability on proper hydrogenosomal function, the metabolic activity of the hydrogenosome and expression of hydrogenosomal enzymes were compared in *Tritrichomonas foetus* maintained under iron-rich (150 μM iron nitrilotriacetate) or iron-restricted (180 μM 2,2-dipyridyl) conditions *in vitro*. The activities of PFOR and hydrogenase, and also production of acetate and H₂, were markedly decreased or absent in iron-restricted trichomonads. Moreover, a decrease in activity of the hydrogenosomal malic enzyme, which is a non-Fe-S protein, was also observed. Impaired function of hydrogenosomes under iron-restricted conditions was compensated for by activation of the cytosolic pathway, mediating conversion of pyruvate to ethanol via acetaldehyde. This metabolic switch was fully reversible. Production of hydrogen by iron-restricted trichomonads was restored to the level of organisms grown under iron-rich conditions within 3 h after addition of 150 μM iron nitrilotriacetate. Protein analysis of purified hydrogenosomes from iron-restricted cells showed decreased levels of proteins corresponding to PFOR, malic enzyme and ferredoxin. Accordingly, these cells displayed decreased steady-state level and synthesis of mRNAs encoding PFOR and hydrogenosomal malic enzyme. These data demonstrate that iron is essential for function of the hydrogenosome, show its involvement in the expression of hydrogenosomal proteins and indicate the presence of iron-dependent control of gene transcription in *Tt. foetus*.

Keywords: *Tritrichomonas foetus*, iron, carbohydrate metabolism, hydrogenosome, transcription

INTRODUCTION

The hydrogenosome is a double-membrane-bound organelle present in trichomonads and other unicellular mitochondrial eukaryotes living in anaerobic or micro-aerobic conditions (Müller, 1993). The organelle was originally described and biochemically characterized in the parasitic flagellate *Tritrichomonas foetus* in the

early 70s (Lindmark & Müller, 1973). Recently, it has attracted the attention of a number of cell evolutionary biologists due to its unusual feature, the production of hydrogen (Martin & Müller, 1998), and its structural and biochemical similarity to mitochondria (Bui *et al.*, 1996; Horner *et al.*, 1996; Biagini *et al.*, 1997; Hackstein *et al.*, 1999). The main recognized function of the hydrogenosome is to generate ATP by means of a metabolic pathway which relies upon the activity of iron-sulfur (Fe-S) proteins: [2Fe-2S] ferredoxin (Johnson *et al.*, 1990), [4Fe-4S] pyruvate:ferredoxin

Abbreviations: Fe-NTA, iron nitrilotriacetate; PFOR, pyruvate:ferredoxin oxidoreductase.

oxidoreductase (PFOR) (Williams *et al.*, 1987) and [4Fe-4S] hydrogenase (Bui & Johnson, 1996). It is likely that other, so far unidentified, Fe-S proteins are present within the hydrogenosome, as indicated by complex EPR spectra (Ohnishi *et al.*, 1980; Chapman *et al.*, 1986). The core hydrogenosomal pathway in which Fe-S proteins are involved utilizes pyruvate and malate as substrates and produces acetate, CO₂ and H₂ (Steinbuechel & Müller, 1986a). Both hydrogenosomal substrates are formed in the cytoplasm as intermediates of glycolytic reactions (Hackstein *et al.*, 1999). In *Tt. foetus*, glycolysis leads to the production of succinate, glycerol and negligible amounts of ethanol, which are excreted (Steinbuechel & Müller, 1986b). Additionally, formate is produced in hydrogenosomes of the anaerobic chytrids (Hackstein *et al.*, 1999). Formation of cytoplasmic pyruvate and its contribution to hydrogenosomal metabolism, however, remains unclear as activity of pyruvate kinase has not been detected in this organism (Hrdý & Mertens, 1993). Malate, formed by cytosolic malate dehydrogenase (Hrdý, 1993), is imported into the hydrogenosome and converted to pyruvate by the activity of an NAD-dependent malic enzyme (Hrdý & Mertens, 1993). PFOR, a key enzyme of the organelle, oxidatively decarboxylates pyruvate to acetyl-CoA. Electrons released during this reaction are transferred via ferredoxin to hydrogenase, producing H₂. The CoA moiety of acetyl-CoA is transferred to succinate and subsequently succinyl-CoA serves as a substrate for succinate thiokinase, which transfers the energy for ATP production (Müller, 1988, 1993).

The importance of Fe-S proteins in the carbohydrate metabolism of trichomonads (Gorrell *et al.*, 1984; Marczak *et al.*, 1983; Müller, 1988; Payne *et al.*, 1993) and other anaerobes (Ellis *et al.*, 1993; Weinbach *et al.*, 1980) may explain the high nutritional requirements of these organisms for iron. It has been shown that addition of 50–100 µM Fe is required for optimal growth of *Tt. foetus in vitro* (Tachezy *et al.*, 1996). Not surprisingly, availability of iron in the cells' environment affects metabolic activity of the hydrogenosome. Gorrell (1985) showed that *Trichomonas vaginalis* had an increased EPR signal for ferredoxin, and also an increased activity of PFOR and hydrogenase if the parasite was grown in a medium with the iron concentration elevated to 200 µM. In *Tt. foetus*, we have demonstrated that activity of PFOR considerably decreased and activity of hydrogenase became undetectable in cells maintained in iron-restricted media in comparison with those grown in an excess of iron (Tachezy *et al.*, 1996). These phenomena could be explained either by a shortage of iron required for assembly of [Fe-S] clusters or by involvement of iron in mechanisms of hydrogenosomal protein expression, a process which is still poorly understood. The hydrogenosomes of trichomonads do not possess their own genome (Clemens & Johnson, 2000). Thus the proteins operating in these organelles are encoded exclusively in the nucleus. Johnson *et al.* (1993) showed that expression of hydrogenosomal proteins closely resembles

that known for the nucleus-encoded proteins of mitochondria. It includes synthesis of nascent protein on free ribosomes (Lahti & Johnson, 1991), protein targeting by means of an amino-terminal leader sequence, translocation to the organelle and intrahydrogenosomal maturation, including cleavage of the leader sequence (Bradley *et al.*, 1997). Whether and at which level (protein targeting, translation, mRNA stabilization, gene transcription) nutritional factors such as iron regulate expression of hydrogenosomal proteins has not been established.

In this paper we provide comparative biochemical data characterizing iron-dependent changes in metabolic fluxes in *Tt. foetus*, we show that iron is involved in expression of both Fe-S and non-Fe-S proteins operating in hydrogenosomes, and we demonstrate that a putative iron-dependent regulation of hydrogenosomal proteins takes place at the transcriptional level.

METHODS

Organism and cultivation. *Tt. foetus* strain Lub-1 MIP (Tachezy *et al.*, 1996) was maintained in trypticase-yeast extract-maltose medium (TYM) supplemented with 10% heat-inactivated horse serum at pH 7.2 (Diamond, 1957). Cells used for comparative studies were subcultured for 10 passages in medium without agar containing either 150 µM iron nitrilotriacetate (Fe-NTA) or 180 µM 2,2-dipyridyl (Sigma).

Cell fractionation. Cytosolic and hydrogenosome-rich fractions used for enzymic studies were obtained by differential centrifugation according to Drmotá *et al.* (1996). To determine the activity of succinate thiokinase, the hydrogenosome-rich fraction was further subfractionated according to Jenkins *et al.* (1991) with a few modifications. The fraction was resuspended in a buffer containing 0.1 M Tris/HCl, 0.175 M KCl and 100 µg leupeptin ml⁻¹, pH 7.5, and sonicated at 10 W for 3 cycles of 5 × 1 s on ice. The sonicated fraction was centrifuged at 130 000 g for 1 h and the supernatant was used for enzyme determinations.

Purified hydrogenosomes, used in the hydrogenosomal protein analysis, were obtained from the cell homogenate using a Percoll gradient according to Lahti *et al.* (1992). At least 90% latency of malic enzyme as a hydrogenosomal marker was found immediately after isolation of the organelles.

Determination of enzyme activities. All enzyme activities were assayed spectrophotometrically at 25 °C. Activities of PFOR (EC 1.2.7.1), hydrogenase (EC 1.18.99.1) and NADH:ferredoxin oxidoreductase (EC 1.18.1.3) were assayed as the rate of methyl viologen reduction monitored at 600 nm. The assays were performed under anaerobic conditions using pyruvate and H₂ as substrates for PFOR and hydrogenase, respectively (Kabičková *et al.*, 1988), and NADH as substrate for NADH:ferredoxin oxidoreductase (Steinbuechel & Müller, 1986a). Activities of adenylate kinase (EC 2.7.4.3) (Dinbergs & Lindmark, 1989), malate dehydrogenase (EC 1.1.1.37) (Drmotá *et al.*, 1997), pyruvate decarboxylase (EC 4.1.1.1) (Čerkasovová *et al.*, 1984), succinate thiokinase (EC 6.2.1.5) (Cha & Parks, 1964) and fumarate reductase (EC 1.3.1.6) were measured at 340 nm under anaerobic conditions as the rate of NADH oxidation. The assay mixture for measurement of fumarate reductase activity consisted of 100 mM sodium phosphate buffer pH 7.5, 10 mM DTT, 500 mM fumarate and 10 mM NADH. The

activity of hydrogenosomal (EC 1.1.1.39) and cytoplasmic (EC 1.1.1.40) malic enzyme was measured aerobically at 340 nm as the rate of NAD⁺ and NADP⁺ reduction, respectively (Drmotá *et al.*, 1996), whilst NADH oxidase (EC 1.6.99.3) was determined as the rate of NADH oxidation (Tanabe, 1979).

Determination of metabolic end products. To determine production of organic acids and alcohols, intact trichomonads were resuspended in Doran's medium (78 mM NaCl, 1.6 mM KCl, 0.6 mM CaCl₂, 30 mM NaH₂PO₄, 15 mM maltose) to a density of 5×10^6 cells ml⁻¹ and 1 ml aliquots were placed into 3 ml vials sealed with vaccine stoppers. The vials were then flushed with nitrogen and incubated at 37 °C for 30 min. After incubation, the cells were removed by centrifugation and the end products determined by HPLC using a PL Hi-Plex H column. HPLC analysis was performed at 65 °C; 5 mM H₂SO₄ was used as the mobile phase. Acetate and succinate were monitored spectrophotometrically at 205 nm, whilst ethanol and glycerol were followed by refractometry.

To determine hydrogen production, the trichomonads were incubated as above for 60 min and hydrogen concentrations measured in the gas phase using gas chromatography. The analysis was performed at room temperature using a molecular sieve 5A column connected to a thermistor detector; nitrogen was used as the carrier gas.

Time course experiment. Trichomonads maintained for 10 passages in the presence of 180 µM 2,2-dipyridyl were resuspended in TYM or 50 mM PBS-maltose (137 mM NaCl, 2.7 mM KCl, 4.6 mM Na₂HPO₄, 1.5 mM KH₂PO₄ and 50 mM maltose, pH 7.2) supplemented with 150 µM Fe-NTA. Aliquots of 1 ml suspension containing 5×10^7 cells were immediately placed into 3 ml vials as above and incubated at 37 °C. Hydrogen production was determined by gas chromatography after various time intervals.

SDS-PAGE and Western blot analysis. SDS-PAGE and Western blotting were used to analyse the protein composition of Percoll-purified hydrogenosomes. SDS-PAGE was performed on a Bio-Rad minislab gel apparatus using 12 or 18 % gel, and 5 % stacking gel. Electrophoretically resolved proteins were stained with silver (Swain & Ross, 1995) or transferred to nitrocellulose membrane to be probed with polyclonal rabbit antibodies against hydrogenosomal malic enzyme (Drmotá *et al.*, 1996), ferredoxin and α -subunit of succinate thiokinase (the latter two antibodies were kindly provided by P. Johnson, UCLA, USA). Quantitative analysis was performed using ScanPack software (Biometra).

Amino acid sequencing. Proteins were separated by SDS-PAGE, transblotted onto a PVDF membrane (Millipore) and stained with Coomassie brilliant blue. The bands of interest were excised and the proteins sequenced from their amino terminus by Edman degradation.

Nucleic acid isolation. Genomic DNA and total RNA were isolated from *Tt. foetus* by modified guanidium thiocyanate methods as described by Bowtell (1987) and Wang & Wang (1985), respectively.

Northern blot analysis. Total RNA was size-separated on 1.2 % agarose/2 M formaldehyde gel and transferred to a nylon membrane (Hybond N; Amersham). Blots were hybridized with radiolabelled DNA probes for hydrogenosomal malic enzyme and PFOR. Subsequently, the membranes were stripped down and rehybridized with DNA probe for β -tubulin as a control.

Nuclear run-on assay. Synthesis of nascent mRNA was investigated by means of a modified run-on assay according to

Ullu & Tschudi (1990). Briefly, trichomonads were washed and resuspended in transcription buffer (150 mM sucrose, 20 mM potassium glutamate, 3 mM MgCl₂, 1 mM DTT, 10 µg leupeptin ml⁻¹, 20 mM HEPES/Tris pH 7.9) and permeabilized in the presence of 1 mg lysolecithin ml⁻¹. The suspension was mixed with the transcription mixture {4 mM ATP, 2 mM GTP, 2 mM CTP, 50 mM creatine phosphate, 1.2 mg creatine phosphokinase ml⁻¹, [α -³²P]UTP [3 kCi mmol⁻¹ (111 TBq mmol⁻¹; Amersham)], 50 mM potassium glutamate, 1.5 mM MgCl₂, 5 mM DTT, 1 mM HEPES} and incubated for 20 min at 35 °C. Nascent RNA was isolated by TRIzol extraction according to the protocol of the manufacturer (Gibco-BRL). ³²P-labelled transcripts were hybridized to DNA-specific probes immobilized on nitrocellulose. Blots were analysed using storage phosphor autoradiography (PhosphorImager SI; Molecular Dynamics) and signals quantified by means of Multi Analyst software (Bio-Rad).

Preparation of DNA probes. All probes were prepared by PCR amplification using specific primer pairs (β -tubulin: CAT CGT CCC ATC TCC AAA GG and AAT GGA ACA AGG TTG ACA GC; malic enzyme: AGG AAG AAC GTG ACC GCC and GTT GCC GAT ATC GTG GTC; PFOR: GAY GGH ACH GTN GGH GC and TCR WAD GCC CAR CCR TC; 16S rRNA: GGT GGT GCA TGG CCG and GTA GGT GAA CCT GCA GAA GGA TCA). For Northern blot analysis, PCR products were purified on agarose gels followed by phenol/chloroform extraction and labelled with [α -³²]dATP using a Random Primed DNA Labelling Kit (Boehringer Mannheim). In the run-on assay, PCR products were inserted into a pGEM vector (TA Cloning Kit; Stratagene) and immobilized on nitrocellulose as a probe. Nucleotide sequences of all PCR products were verified with an automatic ABI Prism 310 gene analyser (Perkin Elmer).

RESULTS

Effect of iron on carbohydrate metabolism

Studies of carbohydrate metabolism revealed that trichomonads are able to switch between cytoplasmic and hydrogenosomal catabolism of pyruvate according to availability of iron. Activities of hydrogenosomal enzymes involved in pyruvate metabolism (malic enzyme, PFOR, hydrogenase, NADH:ferredoxin oxidoreductase) were dramatically reduced in trichomonads grown for 10 subcultures in the presence of iron chelator (180 µM 2,2-dipyridyl) in comparison with those cells maintained under iron-rich conditions (Table 1). Consequently, production of acetate and hydrogen, the major hydrogenosomal end products, was lowered or ceased, respectively, in these organisms (Table 2). It is noteworthy that shortage of iron caused a decrease in enzymic activity for both Fe-S (hydrogenase, PFOR) and non-Fe-S (malic enzyme) proteins. Decreased metabolism of pyruvate in hydrogenosomes was compensated for by increased conversion of pyruvate to ethanol in the cytoplasm. The activity of NADP-dependent malic enzyme and pyruvate decarboxylase increased about twofold and 42-fold, respectively, in the cells grown under iron-restricted conditions. Ethanol became the major metabolic end product in the chelator-treated cells, whilst only a negligible amount of ethanol was produced by trichomonads grown in iron-enriched

Table 1. Effect of iron on enzymic activities involved in carbohydrate metabolism in hydrogenosomes and cytosol of *Tt. foetus*

Enzyme	Iron (150 µM)			2,2-Dipyridyl (180 µM)			2,2-Dipyridyl + iron*		
	Activity†	SEM	n	Activity†	SEM	n	Activity†	SEM	n
Hydrogenosome									
NAD-dependent malic enzyme	236.7	82.5	4	15.0	10.4	10	419.6	22.8	3
PFOR	549.8	362.6	11	50.0	2.0	8	414.2	117.0	4
Hydrogenase	1629.3	798.4	9	0	0	11	4230.3	367.4	3
NADH:ferredoxin oxidoreductase	407.7	161.2	4	44.0	17.7	11	799.0	140.5	3
Adenylate kinase	143.5	32.4	3	118.0	20.2	3	ND		
Succinyl thiokinase	205.2	40.3	4	182.4	47.6	3	ND		
Cytosol									
NADP-dependent malic enzyme	209.4	99.5	12	477.0	183.5	13	199.0	14.0	3
Malate dehydrogenase	3089.0	795.3	3	2217.7	851.0	4	4504.7	355.6	4
Pyruvate decarboxylase	1.0	0	6	42.6	24.1	14	1.0	0	3
Fumarate reductase	165.0	34.8	9	104.0	13.5	14	119.0	5.9	3
NADH oxidase	407.7	161.2	4	44.0	18.7	11	799.0	140.5	3

ND, Not determined.

* 2,2-Dipyridyl (180 µM)-treated cells were transferred to media containing 180 µM 2,2-dipyridyl and 150 µM Fe-NTA for 10 subsequent subcultures before determination of enzyme activities.

† Values are mean specific activities expressed in nmol min⁻¹ (mg protein)⁻¹.

Table 2. Effect of iron on production of cytosolic and hydrogenosomal end products of carbohydrate metabolism

End product	Iron (150 µM)		2,2-Dipyridyl (180 µM)	
	Production*	SEM	Production*	SEM
Acetate	318.4	10.8	51.1	5.6
Hydrogen	27.5	2.2	3.7	1.2
Ethanol	7.5	1.8	950.1	68.6
Glycerol	39.9	4.8	71.0	5.7
Succinate	478.9	20.2	356.6	17.7

* Values are rates of production expressed in nmol (30 min)⁻¹ (mg protein)⁻¹ and are means of four determinations.

media. Availability of iron did not significantly influence activities of the hydrogenosomal enzymes involved in ATP metabolism (adenylate kinase and succinate thiokinase), or of cytosolic fumarate reductase and malate dehydrogenase. Iron-restricted conditions, however, markedly lowered the activity of cytoplasmic NADH oxidase.

To investigate whether the changes in pyruvate metabolism were reversible, the chelator-treated cells were transferred to chelator (180 µM 2,2-dipyridyl)-containing media supplemented with 150 µM Fe-NTA for 10 subsequent subcultures and then the enzymic activities

of interest were determined. As is apparent from Table 1, the activity of hydrogenosomal enzymes increased to comparable or even higher levels than those determined in trichomonads continuously maintained under iron-rich conditions. Conversely, activities of cytoplasmic enzymes involved in pathways leading to production of ethanol (pyruvate decarboxylase, NADP-dependent malic enzyme) decreased after addition of iron. Finally, we monitored the switch from cytoplasmic to hydrogenosomal pyruvate metabolism in a time-course experiment. The chelator-treated cells were resuspended in either nutritionally rich TYM medium containing 180 µM 2,2-dipyridyl or in simple maltose-PBS medium, both supplemented with 150 µM Fe-NTA, and the production of hydrogen as a marker hydrogenosomal end product was monitored for 3 h (Fig. 1). Traces of hydrogen appeared after 1 h and reached a comparable level to that found in chelator-untreated cells after 3 h in TYM medium. Production of hydrogen by cells incubated in iron-containing PBS was also observed. However, it appeared later (after 2 h incubation) and remained considerably lower than that produced by trichomonads incubated in TYM medium.

Iron-dependent changes in protein profile of hydrogenosomes

To determine whether the observed changes in enzymic activities reflect iron-dependent changes in expression of hydrogenosomal proteins, we compared the protein

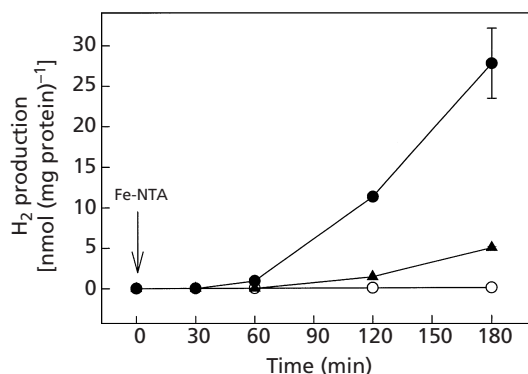


Fig. 1. Stimulation of hydrogen production in iron-deprived *Tt. foetus* after addition of iron. Cells maintained for 10 passages in the presence of iron chelator (2,2-dipyridyl) were resuspended in TYM (●) or maltose-PBS (▲) supplemented with 150 μ M iron as Fe-NTA. Control cells were incubated in TYM with 180 μ M 2,2-dipyridyl (○). After incubation of cell suspensions at 37 °C for defined time periods, production of hydrogen was determined by gas chromatography.

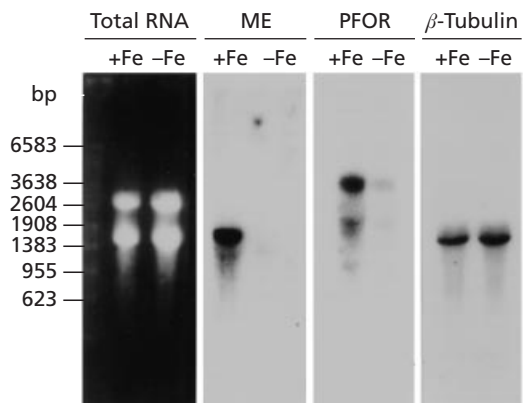


Fig. 3. Comparison of steady-state concentrations of mRNA encoding hydrogenosomal malic enzyme (ME) and PFOR in *Tt. foetus* maintained under iron-rich (+Fe) and iron-restricted (-Fe) conditions. Amounts of total RNA loaded onto each lane were standardized according to the level of β -tubulin mRNA.

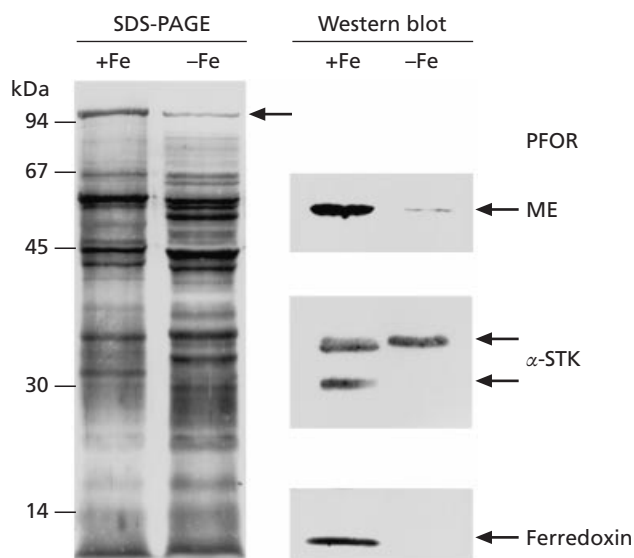


Fig. 2. Iron-dependent changes in profile of hydrogenosomal proteins. Hydrogenosomes were isolated from *Tt. foetus* maintained for 10 subcultures in TYM medium supplemented with 150 μ M Fe-NTA (+Fe) or 180 μ M 2,2-dipyridyl (-Fe). Protein profiles were analysed on 12% acrylamide gels by means of SDS-PAGE and by Western blotting using polyclonal antibodies against hydrogenosomal malic enzyme (ME), ferredoxin and α -subunit of succinate thiokinase (STK). PFOR corresponds to the 120 kDa band on SDS-PAGE gels, as confirmed by amino acid sequence analysis.

profile of hydrogenosomes isolated from trichomonads grown in iron-rich or iron-restricted media. As is apparent from Fig. 2, protein profiles of highly purified hydrogenosomes were markedly different. The amount of a prominent 120 kDa protein corresponding to PFOR

was reduced to about 40% in chelator-treated cells as estimated by densitometry (the identity of this 120 kDa protein was verified by amino acid microsequencing; data not shown). In iron-rich hydrogenosomes, ferredoxin appeared as a 14 kDa band using Western blot analysis (Fig. 2). No comparable band was observed in hydrogenosomes of chelator-treated cells. In addition to differences in levels of Fe-S proteins, we also observed iron-dependent changes in the amount of a protein corresponding to NAD-dependent malic enzyme (60 kDa). The level of malic enzyme was reduced in hydrogenosomes of chelator-treated cells to about 3% of that from iron-rich hydrogenosomes. These results indicate that iron is required for expression of both Fe-S and non-Fe-S proteins involved in hydrogenosomal metabolism of pyruvate.

Consistent with the enzyme activity, antibody against the α -subunit of succinate thiokinase recognized a similar amount of proteins apparent as a 38 kDa band. However, another peptide was identified using this antibody (32 kDa) which was present only in cells maintained under iron-rich conditions. To confirm specificity of the antibody, both bands were analysed by amino acid microsequencing. The analysis showed identity between the N-terminal sequences of the 38 and 32 kDa peptides detected in iron-rich cells and the 38 kDa peptide of chelator-treated organisms (ASGN-LPLLFS). Further iron-dependent differences were observed in the peptides of molecular mass 65, 57, 49, 48 and 33 kDa, which were present in considerably higher concentrations in chelator-treated cells.

Effect of iron on transcription of genes encoding PFOR and malic enzyme

Northern blot analysis was performed to determine whether chelator-treated cells possess reduced levels of steady-state mRNA encoding PFOR and hydro-

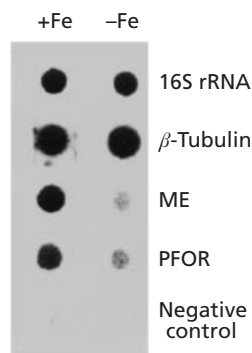


Fig. 4. Transcriptional levels of malic enzyme (ME) and PFOR in *Tt. foetus* maintained under iron-rich (+Fe) and iron-restricted (-Fe) conditions. The levels were measured by nuclear run-on assays using lysolecithin-permeabilized cells. Dot blots contained DNA clones with fragments of 16S RNA, β -tubulin, ME and PFOR genes. The blots were hybridized with nascent 32 P-labelled transcripts. Plasmid pGEM DNA was used as negative control.

genosomal malic enzyme. Consistent with the protein levels, reduced levels of PFOR and malic enzyme mRNA were found (Fig. 3). To exclude the possibility that iron starvation resulted in generalized reduction of mRNAs, the blots were stripped and rehybridized with a probe identifying β -tubulin mRNA. A comparable signal for this mRNA was found in the organisms maintained under both iron-rich and iron-restricted conditions (Fig. 3).

We further measured the synthesis of nascent mRNA encoding PFOR and malic enzyme to determine whether lower mRNA levels resulted from decreased transcription of corresponding genes. The synthesis of mRNA was monitored in detergent-permeabilized cells using a modified run-on assay. As is apparent from Fig. 4, the synthesis of malic enzyme and PFOR mRNA was reduced in the cells maintained for 10 subcultures in iron-restricted medium to about 14 and 27%, respectively. In contrast, no changes in transcription of two control genes (β -tubulin, 16S RNA) were observed in these organisms. The decreased synthesis of malic enzyme and PFOR mRNA, together with observed changes in the steady-state mRNA levels indicate that iron is required for transcription of genes encoding essential proteins involved in pyruvate metabolism within hydrogenosomes.

DISCUSSION

Differences in carbon flow between *Tt. foetus* cells maintained under iron-rich or iron-restricted conditions are summarized in a proposed metabolic map (Fig. 5). Trichomonads grown in excess of iron produced nearly equimolar concentrations of succinate and acetate in the cytoplasm and hydrogenosome, respectively, which is consistent with observations by others (Steinbuechel & Müller, 1986b). In chelator-treated cells, hydrogeno-

somal metabolism dramatically decreased, resulting in a succinate to acetate molar ratio of about 7:1. As expected, decreased acetate production was accompanied by reduced hydrogen production. Conversely, the activities of hydrogenosomal NAD-dependent malic enzyme, PFOR and hydrogenase were low or undetectable in hydrogenosomes isolated from iron-restricted organisms. To compensate for the decreased metabolism of hydrogenosomes, the trichomonads showed increased cytosolic fermentation of pyruvate to ethanol, which became the dominant fermentation end product derived from glucose.

Intriguing questions raised by these observations concern how pyruvate is formed in *Tt. foetus* cytosol and the source of pyruvate serving as a substrate for hydrogenosome metabolism. Although trichomonads metabolize glucose via the Embden–Meyerhoff pathway, leading to formation of pyruvate in *T. vaginalis* (Mertens *et al.*, 1992), activity of pyruvate kinase is either very low or absent in *Tt. foetus* (Hrdý & Mertens, 1993). In both species, pyruvate is alternatively produced from malate which is formed from phosphoenolpyruvate via oxalacetate (Drmotá *et al.*, 1997; Hrdý, 1993). Conversion of malate to pyruvate is catalysed by a cytosolic NADP-dependent malic enzyme (Hrdý, 1993; our unpublished results). Reduced NADP could be regenerated by the activity of an NADP-dependent glycerol-3-phosphate dehydrogenase leading to the production of glycerol (Chapman *et al.*, 1985; Steinbuechel & Müller, 1986b). However, low production of glycerol in *Tt. foetus* observed by us and also by others (7% of fermentation products derived from glucose; Steinbuechel & Müller, 1986b) is not sufficient to account for the redox balance in cells grown under iron-rich conditions. Therefore, we propose that in *Tt. foetus* malate preferentially enters the hydrogenosome where it is converted to pyruvate by the hydrogenosomal NAD-dependent malic enzyme. Contribution of cytosolic pyruvate as the hydrogenosomal substrate seems to be of lesser importance. Similar substrate preference has been found previously in hydrogenosomes of the anaerobic chytrids *Piromyces* sp. E2 and *Neocallimastix* sp. L2 (Hackstein *et al.*, 1999).

Interestingly, malate-dependent formation of pyruvate in the cytosol became essential for carbohydrate metabolism of *Tt. foetus* maintained under iron-limited conditions (Fig. 5). In these cells, oxidative decarboxylation of malate to pyruvate catalysed by NADP-dependent malic enzyme is coupled to the conversion of pyruvate to acetaldehyde mediated by pyruvate decarboxylase. Subsequently, acetaldehyde is reduced to ethanol by alcohol dehydrogenase in a reaction accompanied by reoxidation of NADP, a critical step for maintenance of the redox balance. In addition, production of glycerol slightly increases in iron-restricted cells, which may further contribute to NADP reoxidation.

Iron-dependent modulation of cytosolic/hydrogenosomal carbon metabolism has been observed

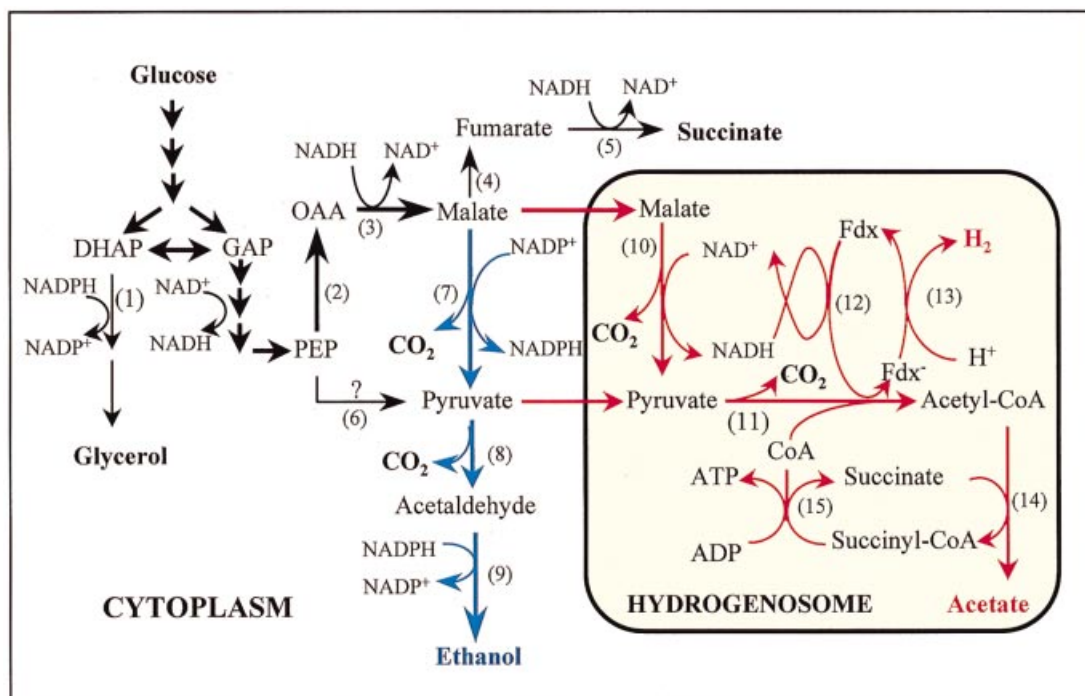


Fig. 5. Iron-dependent changes in carbohydrate catabolism in *Tt. foetus*. Arrows indicate the assumed physiological direction of the reaction. Red arrows indicate the pathways which are active in trichomonads maintained under iron-rich conditions, whilst blue arrows indicate the pathways which are activated under iron-restricted conditions. Bold lines indicate the pathway of the major overall carbon flow. 1, Glycerol-3-phosphate dehydrogenase; 2, phosphoenolpyruvate carboxykinase; 3, malate dehydrogenase; 4, fumarase; 5, fumarate reductase; 6, pyruvate kinase; 7, NADP-dependent malic enzyme; 8, pyruvate decarboxylase; 9, alcohol dehydrogenase; 10, NAD-dependent malic enzyme; 11, pyruvate:ferredoxin oxidoreductase; 12, NAD:ferredoxin oxidoreductase; 13, hydrogenase; 14, acetate:succinate CoA-transferase; 15, succinate thiokinase. (The map was modified with permission of Elsevier Science Publishers from Steinbuchel & Müller, 1986a, b.)

previously in *T. vaginalis* (Gorrell, 1985). Unlike *Tt. foetus*, the human parasite possesses a cytosolic pathway converting pyruvate to lactate, which is a major product of its carbohydrate metabolism. Gorrell (1985) demonstrated that production of lactate decreases, whilst release of hydrogenosomal end products increases, if *T. vaginalis* is maintained in iron-enriched TYM medium.

It is noteworthy that changes in carbohydrate metabolism similar to those caused by iron-restricted conditions in *Tt. foetus* have been observed in trichomonads with *in-vitro*-induced resistance to metronidazole (Čerkasovová *et al.*, 1984; Kulda, 1999). The mode of action of metronidazole, the major drug utilized for treatment of trichomoniasis, resides in its metabolic activation within hydrogenosomes (Müller, 1986). Here, the drug is reduced by ferredoxin-donated electrons which are generated during oxidative decarboxylation of pyruvate or malate. In metronidazole-resistant strains, electrons required for drug activation are not generated as activities of the key enzymes PFOR, malic enzyme and hydrogenase disappear. As in the case of iron-restricted cells, metronidazole-resistant organisms compensate for impaired function of hydrogenosomes by increased rate of cytosolic fermentation to ethanol

(Čerkasovová *et al.*, 1984) or lactate (Kulda *et al.*, 1993). In the present experiments we showed that iron-dependent metabolic changes were fully reversible upon addition of excess iron. Similarly, changes in pyruvate metabolism in the early stages of drug resistance reverted if pressure of metronidazole was released (Kabičková *et al.*, 1988). In light of these observations it is tempting to speculate that mechanisms mediating iron-dependent changes in carbohydrate fluxes and those involved in development of metronidazole resistance in trichomonads may have a related molecular basis.

To determine at which level iron-dependent regulation of hydrogenosomal metabolism takes place, we compared cells of *Tt. foetus* grown under iron-rich and iron-restricted conditions with regard to (i) profiles of hydrogenosomal proteins, (ii) steady-state levels of mRNA encoding PFOR and hydrogenosomal malic enzyme, and (iii) synthesis of nascent mRNAs. Profound changes in protein composition were observed. The levels of two Fe-S proteins, PFOR and ferredoxin, as well as NAD-dependent malic enzyme (a non-Fe-S protein), were markedly decreased in chelator-treated cells. The decreased protein levels of both hydrogenosomal enzymes corresponded to decreased steady-

state levels of pertinent mRNAs on the Northern blots. To distinguish whether the lowered steady-state mRNA levels were due to changes in mRNA stabilization or transcription we performed nuclear run-on assays. These experiments clearly showed that synthesis of nascent mRNA encoding PFOR and malic enzyme is lower in chelator-treated cells. These results indicate that the iron-dependent changes in activity of core metabolic pathways within hydrogenosomes are regulated at the transcriptional level.

Interestingly, Western blot analysis showed differences in the pattern of succinate thiokinase α -subunit (α -STK) isoforms relative to availability of iron, although comparable enzyme activities were detected in cells grown under iron-rich and iron-restricted conditions. It has been shown in *T. vaginalis* that α -STK is encoded by three genes of identical length. However, three bands of different mobility have been recognized using anti- α -STK serum (Lahti *et al.*, 1994). The differences in protein mobility have been ascribed to different post-translational modifications or to conformational differences (Lahti *et al.*, 1994). In our experiments, presence of the lighter peptide (32 kDa) was iron-dependent, whilst the heavier peptide (38 kDa) was produced under both iron-restricted and iron-rich conditions. Alderete *et al.* (1998) identified three *T. vaginalis* genes with either identical (AP51-2) or highly similar (AP51-1, AP51-3) nucleotide sequences known to encode the β -subunit of succinate thiokinase (β -STK). Northern blot analysis has shown that levels of AP51-1 and AP51-3 mRNAs depend on iron availability, whilst the level of AP51-2 mRNA was iron-independent. Thus it is plausible that in trichomonads iron regulates expression of various isoforms of both α -STK and β -STK. Alternatively, iron could be involved in post-translational modification of the proteins, as was demonstrated for the hydrogenosomal malic enzyme (Drmotá *et al.*, 1996).

Iron-dependent changes in enzyme activity have been extensively studied in the Fe-S protein aconitase present in the cytosol of higher eukaryotes. In this enzyme, the loss of enzymic activity under iron-restricted conditions is caused by disassembly of [Fe-S] clusters, while the level of the protein remains unchanged (Yu *et al.*, 1992). Loss of [Fe-S] clusters is associated with an alternative function of aconitase as an iron regulatory protein (IRP) (Kim *et al.*, 1995). Apoconitase mediates post-transcriptional regulation of two molecules central to cellular iron homeostasis, the transferrin receptor and ferritin (Rouault & Klausner, 1997). Recently, a homologue of cytosolic aconitase has been found in the marine ciliate *Eufolliculina ubligi*, although its function as an IRP has not been proven (Markmann-Mulisch *et al.*, 1999). It is not known whether IRPs also operate in trichomonads; however, their presence is unlikely. Trichomonads do not possess either aconitase activity (I. Hrdý, unpublished) or a transferrin receptor (Tachezy *et al.*, 1996). Moreover, our experiments demonstrated the effect of iron on the transcriptional level only. Nevertheless, the presence of iron-dependent post-trans-

criptional regulation cannot be ruled out. Transcriptional control of iron-regulated genes is well-known in bacteria. They possess a histidine-rich protein designated Fur (ferric uptake regulator) which acts as a repressor together with iron (Crosa, 1997; Hantke, 1984). The main function of Fur is to turn off the expression of genes involved in iron uptake under iron-rich conditions. To our knowledge, iron-dependent control of protein expression of the type reported here for trichomonads is different from other mechanisms of iron-dependent regulation described so far. In trichomonads, iron is involved (i) in positive transcriptional control, and (ii) in regulation of expression of proteins involved in core energy metabolism. Further studies are required to obtain detailed information on the molecular mechanisms of the iron regulatory system of trichomonads, which might be unique to these unicellular eukaryotes.

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