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Characterization of flavodiiron protein from *Trichomonas vaginalis* hydrogenosomes

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I declare that I have prepared my thesis on my own and I have presented all the references used.

Tamara Smutná

Contents

1. List of abbreviations

ADP	adenosin diphosphate
AP	alkaline phosphatase
β-NAD	β-nicotinamide adenine dinucleotide
BSA	bovine serum albumin
CBB	Coomasie Brilliant Blue R 250
CoA	coenzyme A
Complex I	homologue of the NADH dehydrogenase module of mitochondrial Complex I [EC 1.6.5.3]
DAPI	4'-6-diamidino-2-phenylindole
DCIP	dichlorophenolindophenol
DNTP	deoxyribonucleotide triphosphate
ECL	enhanced chemiluminiscence
EDTA	ethylenediaminetetraacetic acid
FAD	flavin adenine dinucleotide
FC	Folin-Ciocalteu's Reagent
FDPs	flavodiiron proteins
FIRd	flavorubredoxin
FIRd-Red	flavorubredoxin reductase
FMN	flavin mononucleotide
FOO	ferredoxin:oxygen oxidoreductase
FOO His	FOO protein with 6x histidine tag
FOO HaHa	FOO protein with haemagglutinin tag (2x haemagglutinin)
FOO WT	FOO protein without tag
HPLC	High Performance Liquid Chromatography
Hrb	high molecular weight rubredoxin
hydrogenase	hydrogen:ferredoxin oxidoreductase [EC 1.12.7.2]
IM	isolation medium
IPTG	isopropyl-ß-D-thiogalactopyranosid
LGF	large granule fraction
ME	malic enzyme, [malate dehydrogenase (decarboxylating), EC 1.11.3.9]
MerOH	mercaptoethanol

MN	transfection vector MasterNEO
MN *FOOHaHa*	transfection vector MasterNEO containing gene for FOO protein with HaHa tag on C-terminus
MN *FOOHis*	transfection vector MasterNEO containing gene for FOO protein with His tag sequence on C-teminus
MN *FOOStrep*	transfection vector MasterNEO with *FOO* gene with *Strep*-tag
MN *FOOWT*	transfection vector MasterNEO containing gene for FOO protein
MV	methylviologen
NADH	nicotinamide adenine dinucleotide – reduced form
NRO	NADH:rubredoxin oxidoreductase
OD	optical density
PBS	phosphate buffered saline
Pd	palladium
PFO	pyruvate:ferredoxin oxidoreductase [EC 1.2.7.1]
Ponceau S	3-hydroxy-4-[2-sulfo-4-(-sulfophenylazo)phenylazo]-2,7-naphalenedisulfonic acid
Rf	retention factor
Rd	rubredoxin
ROO	rubredoxin:oxygen oxidoreductase
ROS	reactive oxygen species
RT	room temperature
SA	specific activity
SDS-PAGE	Sodium dodecyl sulfate – polyacrylamide gel electrophoresis
STK	succinate thiokinase
TEMED	N,N,N',N'- tetramethylethylenediamine
TLC	thin layer chromatography
TLCK	tosyl-lysin-chlormetylketon
Tris	tris[hydroxymethyl]aminomethane; TRIZMA base
TV3	plasmid TagVag 3 containing *Strep*-tag
TvFdx	*Trichomonas vaginalis* ferredoxin
TvFOOHaHa	*T. vaginalis* line overexpressing FOOHaha
TvFOOHis	*T. vaginalis* line overexpressing FOOHis
TvFOOStrep	*T. vaginalis* line overexpressing FOOStrep

TvFOOWT	*T. vaginalis* line overexpressing FOOWT
TvT1	*Trichomonas vaginalis* strain T1
TYM	Diamond's medium
Tween 20	polyoxiethylenesorbitan monolaureate
TX 100	Triton X-100
X-Gal	5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside

2. Introduction

2.1 *Trichomonas vaginalis*

Trichomonas vaginalis is a microaerophilic parasitic protozoon responsible for trichomoniasis in humans [1].

This is the most prevalent non-viral sexually transmitted infection, with estimated over 170 million cases occurring each year [2], and has been implicated as a major risk factor in a predisposition to human immunodeficiency virus / AIDS [3].

Infection with *T. vaginalis* is also recognized as contributing to low birth-weight and preterm delivery [4]. Acute human trichomoniasis, characterized by vaginitis with erosions of vaginal mucosa and purulent discharge, causes extreme discomfort to patients. Although the symptoms usually cease with transition of the disease into the chronic or latent phase, the infection is not self-limiting and exacerbations of acute episodes are common [5].

Diseases caused by trichomonads can be cured by 5-nitroimidazole drugs, such as metronidazole, effective against a variety of anaerobic protozoa and bacteria. The antimicrobial effect of these drugs depends on their metabolic reduction within the target cell resulting in the release of reactive free radicals [6].

This parasite itself is an unusual eukaryote. Biochemically, it is a fermentative organism gaining carbon and ATP by anaerobic breakdown of glucose, and, in vivo, also of aminoacids, namely arginine [7].

T. vaginalis has been considered to be one of the earliest branching eukaryotic organisms known, [8] although current evidence for this view is not compelling [9-11]. It lacks conventional mitochondria but possesses organelles termed hydrogenosomes that share common ancestry with mitochondria [12,13] and appear to be adaptations for the parasite`s existence in an environment containing only low oxygen concentrations.

The life cycle includes only trophozoite stage proliferating by binary division.

2.1.1 Structure of trichomonad cell

T. vaginalis is a eukaryotic organism with characteristic cell composition and size of about 15 x 7 μm [14]. The shape of the cell changes from spheroidal to amoeboid. *T. vaginalis* cell is surrounded by typical plasma membrane of and is equipped with 5 flagella, one of which is recurrent (Fig.1, page 8).

The recurrent flagellum is attached to the cell and together with accompanying structures forms a more complex motoric organelle – the undulating membrane.

Flagella with their basal bodies, the flagellar roots attached to the basal bodies, and the centrosomal structures associated with the basal bodies comprise characteristic mastigont of trichomonads [15]. Microtubular structures of the mastigont are axostyle and pelta constituting an axial skeleton and subpelicullar endorsement of the exposed apical area where the flagella emerge out of the cell. Trichomonads have conspicuous striated root fibrils – the costa serving as mechanical support of the undulating membrane and the parabasal fibrils associated with the Golgi complex forming so-called parabasal apparatus. From the two types of costae present in trichomonads, A and B, *T. vaginalis* possess B type with characteristic design of striation and attachment to kinetosomes [16].

The axostyle of trichomonads is noncontractile. The axostylar sheet of interconnected parallel microtubules is rolled into hollow cylindrical trunk. In the direction of apical part of the cell it opens in a nuclear area to form a spoon-like capitulum, where the nucleus is situated.

The nucleus of trichomonads is haploid and possesses 6 telocentric chromosomes [17, 18]. Trichomonads multiply by binary division with a specific type of mitosis characterised by the presence of extranuclear spindle [19] and retained nuclear membrane.

As already mentioned, there are no mitochondria in trichomonads; instead, they have numerous organelles specific to certain anaerobic protozoa – the hydrogenosomes, which accumulate in perinuclear, axostylar and subcostal areas [20].

The cytoplasm contains variety of vacuoles and coated vesicles relative to endocytosis, digestion and transport, rough endoplasmatic reticulum, free ribosomes and polysomes, and glycogen granules. Glycogen, the main endogenous fuel of trichomonad glycolysis, is abundant in trichomonad cytoplasm in the form of α – rosettes [16,21,22].

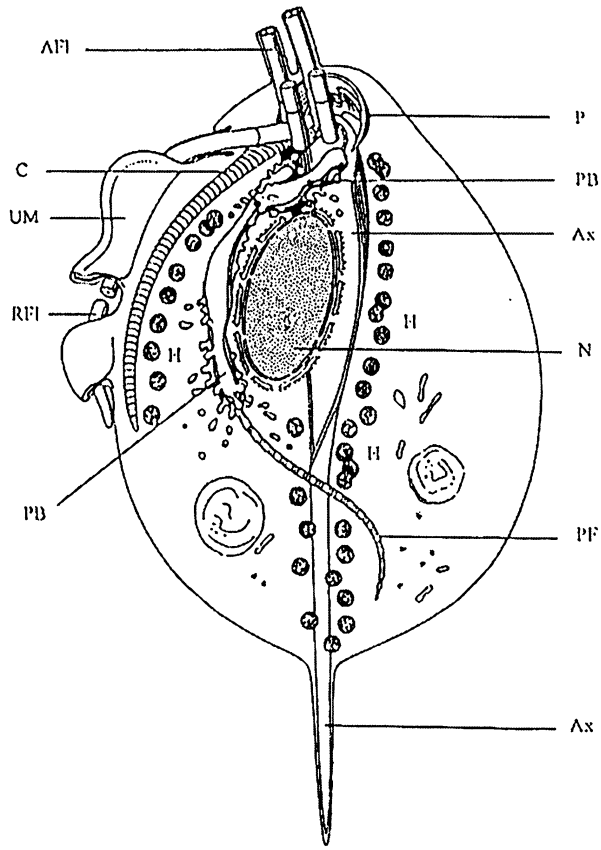


Figure 1. Schematic diagram of *T. vaginalis* cell.

AFl: anterior flagella, C: costa, P: pelta, N: nucleus, PB: parabasal apparatus (modified Golgi complex), PF: parabasal fibril, Ax: axostyle, H: hydrogenosome, UM: undulating membrane, RFl: recurrent flagellum [23].

2.1.2 The hydrogenosome

Trichomonas vaginalis lacks morphologically recognizable mitochondria but contains another type of membrane-bounded organelle, which was named the hydrogenosome [20,24-27]. These characteristic organelles are predominantly spherical or somewhat elongated structures, about 0.5 μm in diameter [28], and occupy about 6% of the cell volume [28]. They are bounded by an envelope consisting of two closely apposed unit membranes [29,30], and contain a granular matrix and often an electron-dense core (Fig.2, page 10).

New hydrogenosomes are generated by division of preexisting organelles and do not bud from the endoplasmic reticulum [31]. The hydrogenosome may divide by means of two processes: segmentation and partition, as previously described for mitochondria [32-34], pointing to additional similarities between the two organelles. An intimate association of the hydrogenosome with the endoplasmic reticulum (ER) was observed. As the hydrogenosome enlarges before its division, new membranes have to be formed. The ER could participate by providing the membrane lipids [31].

In contrast to mitochondria, DNA has not been detected in the hydrogenosomes of trichomonads [35-37]. Hydrogenosomal proteins of *T. vaginalis* are encoded by nuclear DNA and synthesized on free polyribosomes, released in the cytoplasm and subsequently translocated into the organelle [38]. Proteins directed to the organelle contain amino-terminal, cleavable sequences which operate as targeting signals [39]. However, there are also hydrogenosomal proteins without any clear targeting sequence [40]. Interestingly, hydrogenosomal amino terminal targeting sequences are similar to mitochondrial ones. They both have an amino-terminal location, are cleaved from mature protein found in the organelle and have similar amino acid composition. The major difference between these sequences is in the length. The amino terminal presequences of yeast and mammalian mitochondrial proteins contain typically 20-80 amino acids, while hydrogenosomal presequences are typically 5-13 amino acids long. Specific amino acids are in most targeting sequences in conservative positions, as, for example, leucine in the second position from N-terminus [41]. Arginine is present at either –2 or –3 from the cleavage site, and asparagine or phenylalanine is present at –1 from the cleavage site. These sequences are also rich in hydrophobic and hydroxylated amino acids [42]. Targeting sequences of hydrogenosomes, like mitochondrial ones, are able to create amfifilic α-helixes [43,44].

Examples of hydrogenosomal targeting sequence:

Hydrogenosomal malic enzyme (ME) gene A and β-subunit of succinyl-CoA synthase (β-SCS 1):

ME A	M L T S S V S V P V R N
β-SCS 1	M L S A S S N F A R N

X –amino acid in conservative position

Transport of proteins into hydrogenosome requires electrochemical gradient and ATP [42], and membrane transporters participating on protein transfer are sensitive to treatment with trypsin [45].

The hydrogenosome is defined by its unusual function: under anaerobic conditions it produces molecular hydrogen by reduction of protons using electrons released by oxidation of pyruvate or malate. More recently, organelles with similar biochemical properties have been found in a number of additional protist groups, all from oxygen-poor environments: certain free-living [46-48] and rumen ciliates (*Ciliata*) [49-53] and chytrid fungi (*Chytridiomycota*) [54,55]. The presence of hydrogenosomes in additional protist groups is likely. No hydrogenosomes have yet been found in multicellular animals or plants.

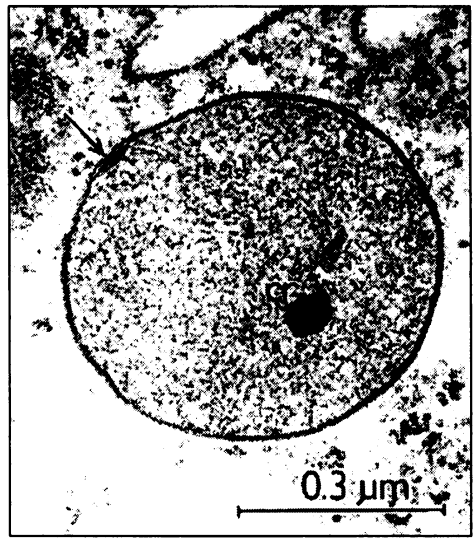


Figure 2. Hydrogenosome (gently provided by J. Kulda).
cr - electrondense core, arrow – intermembranous vesicle

2.2 Biochemistry of hydrogenosome

Hydrogenosomes present in *T. vaginalis* generate ATP by the fermentative conversion of malate and pyruvate to acetate, CO_2 and molecular hydrogen [56] (Fig.3, page 13).

Pyruvate is either formed by glycolysis and enters the organelle or is produced directly in hydrogenosome by oxidative decarboxylation of malate [57,58]. In the next step, pyruvate is oxidatively decarboxylated. The enzyme responsible for pyruvate oxidation, a key enzyme of organelle, has been identified as pyruvate:ferredoxin oxidoreductase (PFO) in several species [20,24,25]. This membrane-bound enzyme serves the same role as pyruvate dehydrogenase (PDH) complex in mitochondria [59,60]. It is a homodimer of two 120 kDa subunits and contains binding site for thiamine pyrophosphate and [4Fe-4S] iron-sulphur centres (but no lipoate or lipoamide). This oxygen-sensitive enzyme catalyzes oxidative decarboxylation of pyruvate with the formation of acetyl CoA and CO_2 [20, 59].

The CoA moiety of acetyl-CoA is subsequently transfered to succinate, forming succinyl-CoA. The reaction is catalyzed by enzyme acetate:succinate CoA transferase. The energy of thioester bond in succinyl-CoA serves as substrate for a substrate-level phosphorylation producing ATP by succinate thiokinase [61].

Reducing equivalents derived from oxidation of pyruvate are transferred to protons with the formation of H_2 under anaerobic conditions [56]. In summary, the result of pyruvate metabolism in hydrogenosome is 1 molecule of ATP per one molecule of pyruvate. As final products, acetate, H_2 and CO_2 are released [56].

The dominant electron transporter involved in hydrogenosomal redox reactions appears to be ferredoxin. Hydrogenosome completely lacks heme proteins and ferredoxins represent its main electron transferring components [62]. These small iron-sulfur proteins exist in nature in several forms, which are characterised by organisation of Fe-S centre and wide spectrum of redox potentials. Hydrogenosomal ferredoxin of *T. vaginalis* contains [2Fe-2S] [63] centre and its redox potential is –347 mV [64]. The presence of [2Fe-2S] centre in ferredoxin and structure of the protein distinguishes trichomonads from other anaerobic protozoa (*Giardia intestinalis*, *Entamoeba histolytica*) utilizing ferredoxin transporters. These parasites possess different 2[4Fe-4S] ferredoxins, typical representatives of another subgroup of these proteins. Trichomonad

ferredoxins are related to ferredoxins present in aerobic bacteria (e.g. *Pseudomonas putida* and others) and in mitochondria [65].

The presence of the NADH dehydrogenase components of mitochondrial-type respiratory complex I in *Trichomonas* hydrogenosomes fills important gap in the redox balance of hydrogenosome metabolism [66,67]. The enzyme can replenish the NAD^+ pool by reoxidizing NADH produced by the hydrogenosomal malic enzyme. It catalyses the previously elusive NADH:ferredoxin oxidoreductase activity, to provide an alternative source of reducing power to that provided by pyruvate:ferredoxin oxidoreductase [56].

Reoxidation of ferredoxin in the hydrogenosome may proceed by transfer of electrons to protons by hydrogenase. Hydrogenases of trichomonads are oxygen-sensitive enzymes which utilize reduced ferredoxin as an electron donor [20,24,25]. Hydrogenase of *T. vaginalis* participating in reduction of ferredoxin was partially purified [68] and characterized as an Fe-only hydrogenase [68]. This type of hydrogenase is present in anaerobic bacteria such as *Clostridium pasteurianum* and *Desulfovibrio vulgaris* [69,70].

Under aerobic conditions the organelles also produce CO_2 and acetate and phosphorylate ADP to ATP [56]. There is no H_2 production, however, and oxygen is consumed by the organelle [71,72]. The likely product of O_2 reduction is $O_2^{-\cdot}$ and subsequently H_2O_2 [56]. The enzyme possessing oxidase activity is not susceptible to inhibitors of mitochondrial respiration [71] and remains to be identified.

Enzymes of the tricarboxylic acid cycle [73] as well as cytochromes, cytochrome oxidase [74] and F_0F_1 ATPase [75] have not been detected in trichomonad hydrogenosomes with the exception of succinate thiokinase. The absence of all these typical mitochondrial proteins has now been verified by the recently completed *T. vaginalis* genome-sequencing project (http://www.tigr.org/tdb/e2k1/tvg/).

Hydrogenosomal metabolism has important implications for chemotherapy of trichomoniasis. The drugs of 5-nitroimidazole group, such as metronidazole, are activated in hydrogenosome to reactive cytotoxic intermediates – nitro radical anions. Metronidazole enters the cell and the organelle by simple diffusion. In hydrogenosome it acts as a preferential electron acceptor, competing efficiently for electrons with the hydrogenase. Consequently, the production of hydrogen is inhibited [76] and ferredoxin-mediated transport of electrons is directed to the drug. The development of anaerobic metronidazole resistance (in vitro) is coupled with the consecutive loss of

pyruvate:ferredoxin oxidoreductase and hydrogenosomal malic enzyme activities. These enzymes provide electrons for ferredoxin which reduces metronidazole to its toxic nitro-radical. The enzyme hydrogenase, terminal acceptor of electrons in drug – susceptible trichomonads, and electron carrier ferredoxin are other hydrogenosomal proteins that disappear in highly drug – resistant trichomonads [5].

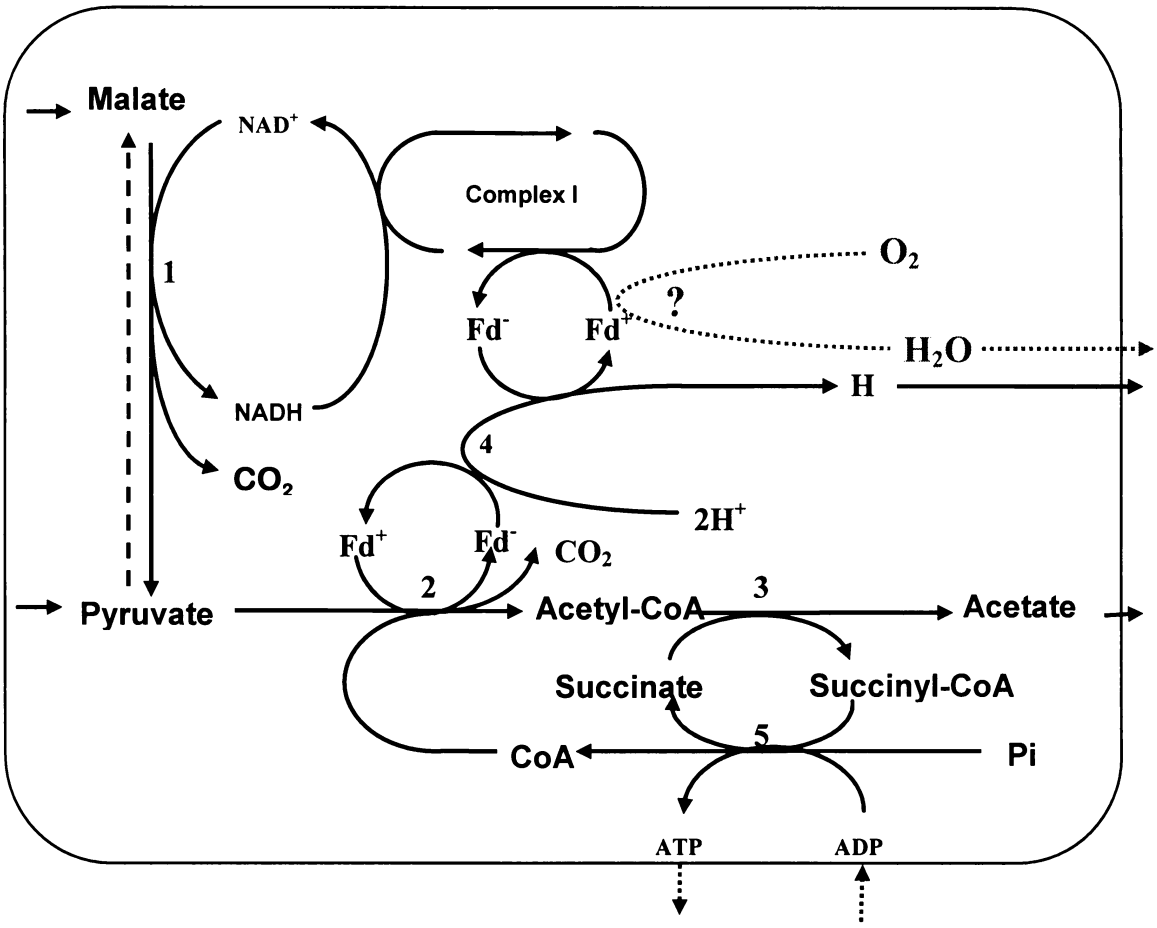


Figure 3. Metabolism of hydrogenosome.

1, malate dehydrogenase (decarboxylating) (malic enzyme); **2**, pyruvate:ferredoxin oxidoreductase; **3**, acetyl-CoA:succinate CoA transferase; **4**, hydrogenase; **5**, succinate thiokinase; Complex I, homologue of the NADH dehydrogenase module of mitochondrial complex I with NADH:ferredoxin oxidoreductase activity; **?**, ferredoxin:oxygen oxidoreductase (FOO) [109].

2.3 *Trichomonas vaginalis* and oxidative stress

All aerobic organisms are exposed to reactive oxygen species (ROS) such as superoxide anions ($O_2^{-\cdot}$), hydrogen peroxide (H_2O_2) and hydroxyl radicals ($OH^{\cdot}$) generated by their metabolism.

Parasitic protozoa not only have to eliminate their endogenous toxic metabolites but they must also cope with the oxidative (or respiratory) burst of the host immune system. During recent years, several antioxidant and detoxification systems of parasitic protozoa have been identified and their role in the survival of the parasites investigated [77].

Some of the parasites` enzymes are inactivated by oxygen itself, notably key proteins of the hydrogenosome (hydrogenase, PFO), and various metabolites likely to arise from the metabolism of oxygen (such as hydrogen peroxide and hydroxyl free radical) are generally harmful to cells and so need to be countered. Most eukaryotes have glutathione as a key redox buffer and antioxidant but trichomonads seem to lack this and similar thiols [78]. Cysteine has been considered the main cellular reducing agent and antioxidant, although *T. vaginalis* is able to generate thiols (propanethiol, methanethiol, and hydrogen sulfide), which have been postulated to have antioxidant roles [78], from the action of the unusual bacterial - like enzyme methionine–γ–lyase [79].

T. vaginalis relies on cytosolic NADH oxidase (reducing oxygen to water) and cytosolic NADPH oxidase (reducing oxygen to hydrogen peroxide) to prevent permeation of oxygen to the hydrogenosomes. The production of H_2O_2 by NADPH oxidase and also by superoxide dismutase poses the question of how are these and other ROS scavenged because *T. vaginalis* lacks catalase and glutathione - dependent peroxidase activities [77].

A family of peroxidases has been discovered in recent years that do not use glutathione as the reductant but instead are dependent on reduction by a small dithiol protein known as thioredoxin (Trx), which is itself reduced by thioredoxin reductase (TrxR) [80-82]. These Trx - dependent peroxidases, now commonly known as peroxiredoxins (designated TrxP), are seemingly ubiquitous in eukaryotes [82-86]. It has been shown that peroxiredoxins reduce hydrogen peroxide and alkyl hydroperoxides and therefore play an important role in cellular protection system against the devastating consequences of oxidative damage [80,86].

The data presented for the recombinant TrxR, Trx and TrxP of *T. vaginalis* suggest that these three proteins could reduce hydroperoxides resulting from oxidative stress. The increased expression of both Trx and, in particular, TrxP when *T. vaginalis* was experimentally exposed to oxidative stress is fully consistent with a role in enabling the parasite to with-stand such challenges [87].

Recently, two proteins, rubrerythrin and a peroxiredoxin of the bacterial periplasmic Tpx – type [86] have been identified in the proteome of hydrogenosomes of *T. vaginalis*. These proteins are candidates for peroxide scavenging in hydrogenosomes [40].

2.4 Flavodiiron proteins (FDPs)

The flavodiiron proteins (first named as A-type flavoproteins) [88] constitute a large superfamily of enzymes, widespread among anaerobic and facultative anaerobic prokaryotes, from both the Archaea and Bacteria domains. Notably, genes encoding for homologous enzymes are also present in the genomes of some parasitic and anaerobic amitochondriate protozoa [88,89]. The fingerprint of this enzyme family is a conservation of a two-domain structural core, built by a metallo-β-lactamase-like domain, at the N-terminal region, harbouring a non-heme diiron catalytic site, and a flavodoxin-like domain, containing one FMN moiety [89].

The first function assigned to these enzymes was that of *Desulfovibrio gigas* enzyme, which was shown to reduce oxygen to water, receiving electrons from rubredoxin [90, 91]; therefore it was named rubredoxin:oxygen oxidoreductase (ROO).

2.4.1. Classification of flavodiiron proteins

The flavodiiron proteins have in common the two-domain structural core. However, several members of this family have extra domains, fused at the C-terminus (Fig. 4, page 16). Thus, according to the domain composition, these enzymes can be divided into three classes, A to C:

Class A, having only the two domain core, which so fare includes the largest number of members.

Class B, containing a rubredoxin-like domain with an incorporated FeCys4 binding motif, similar to those of Type I rubredoxins. Genes encoding for class B enzymes are so far found only in the genomes of enterobacteria (which contain only this type of flavodiiron proteins) and in *Erwinia chrysanthemi* [92].

Class C, which have an additional module (of ca. 170 residues) with significant similarities to NAD(P)H:flavin oxidoreductases, and only detected in cyanobacteria. Within this class, two subgroups can be distinguished, according to the conservation of the iron-binding residues.

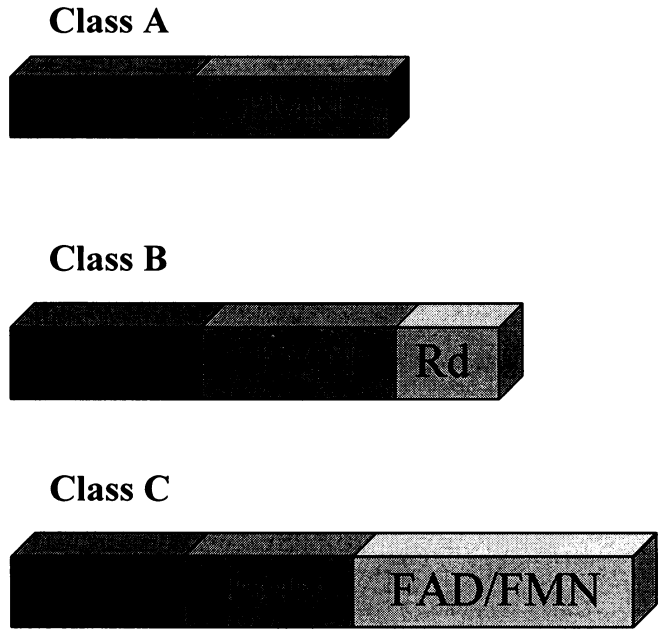


Figure 4. Modular arrangement of the different classes of flavodiiron proteins [93].

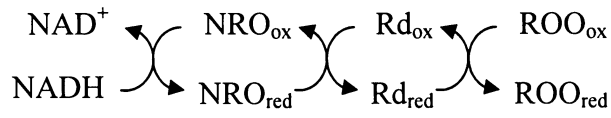
The Class A FDPs, which correspond to the flavodiiron structural core, have a length of approximately 400 amino acids, and thus a molecular mass around 45 kDa; Class B and Class C FDPs have higher molecular masses – around 54 kDa and 65kDa, respectively – due to the extra C-terminal modules. With the exception of the *M. thermoautotrophicus* Marburg protein (homotetramer), the quarternary structure of FDPs is that of a homodimer. The monomers are organized in the head-to-tail position placing the FMN and diiron centres from the opposing monomers within efficient electron transfer distance [93].

2.4.1.1. Class A enzymes

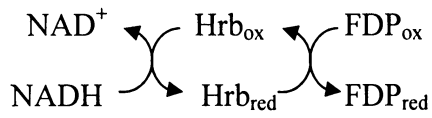
Desulfovibrio gigas ROO has been shown to accept electrons directly from the one electron-reduced rubredoxin (Rd) partner [90,91]. The rubredoxin, a small redox protein of approximately 6 kDa with a Fe-S(Cys)$_4$ centre, is in turn reduced by an NADH:rubredoxin oxidoreductase (NRO) [94]. This electron transfer chain provided the first clear example of a function for a rubredoxin in anaerobes. In contrast to another recently proposed mechanism for oxygen detoxification in anaerobes involving the intermediary formation of a superoxide anion and hydrogen peroxide [95], ROO provides both oxygen scavenging and energy conservation mechanisms; the direct reduction of dioxygen to water avoids the production of above mentioned reactive oxygen species [89].

In *Moorella thermoacetica*, the C-terminal modules from Class B and class C flavodiiron proteins, i.e. the Rd and NAD(P)H:flavin oxidoreductase modules, are fused together in a single polypeptide chain, which was named by the authors as a high molecular weight rubredoxin (Hrb). The genes encoding for both Hrb and the flavodiiron protein are part of the same operon, which includes also a putative oxidative stress response protein, rubrerythrin [96].

The Hrb NADH:flavin oxidoreductase module was proposed to accept electrons from NADH and internally transfer them to the Rd module, which acts as the donor site for the flavodiiron protein [97] (Fig.5).



Desulfovibrio gigas



Moorella thermoacetica

Figure 5. Scheme of FDPs reduction in Class A. Transport of electrons in *Desulfovibrio gigas* and *Moorella thermoacetica* [93].

2.4.1.2. Class B enzymes

In *E. coli*, the flavodiiron protein, flavorubredoxin, was shown to interact directly with an NADH–dependent (flavo)rubredoxin reductase (FIRd-Red) [98] (Fig.6).

The direct reduction of the terminal enzyme component FIRd by an NADH-dependent partner was a result of an interesting evolutionary event, which fused the Rd component to the flavodiiron structural core. It was determined, that the flavorubredoxin reductase is a 43 kDa monomeric NADH oxidase, containing one FAD, which efficiently reduces FIRd [98].

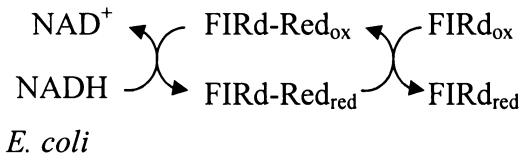


Figure 6. Reduction chain of FDPs in Class B [93].

2.4.1.3. Class C enzymes

An even more extreme module fusion is observed in the cyanobacterial flavodiiron proteins, both in the studied member of *Synechocystis* sp. PCC 6803 [99] and the remainder multiple copies found in all the cyanobacterial sequenced genomes [100, 101]. In this case, the multi-component electron transfer chains are completely abolished, as the fusion of the NAD(P)H:flavin oxidoreductase module to the flavodiiron core allows the protein to accept electrons directly from NAD(P)H and perform several intra-molecular electron transfer steps onto the diiron centre, which ultimately reduces the diatomic substrate (Fig. 7).

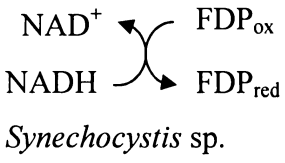


Figure 7. Mechanism of reduction FDPs in Class C [93].

2.4.2. Function of flavodiiron proteis

The first function assigned to a flavodiiron protein was that of oxygen reduction to water, for the enzyme from *Desulfovibrio gigas*. The same function was later reported for the enzymes from *E. coli* and *Synechocystis* sp. Already in 1998 it was proposed that *E. coli* possessed a nitric oxide reductase activity, when grown anaerobically, which was insensitive to cyanide, thus excluding flavohemoglobin as the responsible enzyme [102]. Later on, Gardner at al. [103] showed, based on several molecular genetics studies, and on the fact, that the diiron site of *E. coli* flavorubredoxin was able to bind NO [98], that flavorubredoxin was NO reductase.

More recently, the recombinant enzyme from *M. thermoacetica* was also shown to reduce NO, again in the presence of NADH and its physiological partner, the high molecular weight rubredoxin, Hrb [97].

The product of NO reduction by FDPs has not been demonstrated yet. Nevertheless, the NADH/NO stoichiometry and the reaction kinetics strongly suggest that the product will be N_2O [97]. Although this gas is essentially harmless, and may be eliminated by diffusion, it is also possible that an unknown N_2O reductase is present in these organisms.

A major question regarding these enzymes is whether the initially proposed activity as oxygen reductases is physiologically relevant. The affinity for oxygen is much lower than that for NO [97,104], indicating that nitric oxide is the preferred substrate. But, as discussed for the (flavo)hemoglobins, it may also be that these enzymes are bifunctional, being active both in oxidative or nitrosative stress conditions. In fact, binuclear iron centres in multiple enzymes are known to reduce oxygen to water, when the enzymatic reaction is decoupled from the substrate, and bind nitric oxide, which is usually used as a probe for diiron centres. This situation finds also a parallel in the heme-iron NO reductases and the heme-copper oxygen reductases, which although having a much higher affinity for their natural substrate, are nevertheless capable of reacting with oxygen or nitric oxide, albeit with a much lower turnover [105-108].

2.5 Ferredoxin:oxygen oxidoreductase (FOO)

Ferredoxin:oxygen oxidoreductase is a flavoprotein recently discovered in *T. vaginalis*. This protein was detected coincidentally during the search for the hydrogenosomal homologue of mitochondrial Complex I by RNDr. I. Hrdý, PhD. and J. Vašák (Department of Parasitology, Charles University in Prague). Protein sequence analysis (Fig. 8) revealed clear homology of the new protein with the recently established family of flavodiiron proteins [93], namely with ROO. Because *T. vaginalis* lacks rubredoxin, but possesses multiple ferredoxin genes, we supposed that ferredoxin could be an electron donor to our flavoprotein and in analogy to ROO we decided to preliminarily name it ferredoxin:oxygen oxidoreductase.

MLSTSSARSFSALDLGVTKVADDVLWIGVNDWDLREFHSMQSPVG
TSYNSYLIQSSEPTIVDAVKYTMAHAWIDRLKSIGGDDLKGIKRI
IVQ■A■P■HSSGTAMLVKEAPHIEVVMTKQCYNTMARFYDVSKWN
VKIVKLGEKINLGDRVAVMAGVPMA■WPESAVTYLPAQKILFSS■
CFGQHIASNKRFVDEVDQGLFLTEIKSYYANILQRLGKPVLKALA
TASKLPGLDIVLPA■GVGFRRKEDLEQAIKLYTQWATYKPNPKVS
■■■■DCNWYG■EKM■EA■ASGICKEKLECIMMNARKTHITRSATEV
MDSAAVAFGSSTLHECILPDIAMHLNYLRCLGIHDKPVGLFGTFG
WQEKIVPNELRNAIVKPCKIPEVAEPVMAHWNPKEADLAKCEELG
RKLALAAIEKCK

Figure 8. FOO aminoacid sequence.
X, hydrogenosomal leader sequence; ■, iron ligands; underlined residues denote flavodoxin signature sequence; ■, highly conservative positions in flavodoxin signature sequence.

Flavodoxin signature:
[LI■]-[L■VFY]-[F■]-X-[ST]-X-X-[AGC]-X-■-X-X-X-■-X-X-[LI■]
green, aminoacids matching FOO flavodoxin signature; X, arbitrary aminoacid

3. Aims

My master thesis project, performed at the Department of Parasitology, Faculty of Science, Charles University in Prague, was to characterize the recently discovered flavoprotein in the parasitic protozoon *Trichomonas vaginalis*.

This protein was supposed to have an oxygen-scavenging function in the hydrogenosomes of this oxygen-sensitive organism. Following the assumption, that ferredoxin may serve as electron donor for this protein, we named it ferredoxin:oxygen oxidoreductase (FOO).

In my work, I have attempted to address the following tasks:

- To determinate the native molecular weight of FOO protein and the type of flavine cofactor contained in its molecule.
- To locate the FOO protein in *Trichomonas vaginalis* cell.
- To prepare *Trichomonas vaginalis* strains overexpressing FOO in order to study the phenotype differences that could point to the physiological role of the FOO.
- To verify the level of expression of FOO in metronidazole-resistant *T. vaginalis*.

4. Materials and methods

4.1 Stock solutions and buffers

- Antibiotics:

  Ampicilin (Sigma).....100 mg/ml

  Chloramfenicol (Sigma).....50 mg/ml

  Geneticin (G418) (Sigma).....200 mg/ml

  Penicillin (Biotika).....100 000 U/ml

- Antibodies:

  Primary antibodies:

  anti- TvH47 (Complex I subunit) polyclonal serum (rabbit); 1:1000 [109]

  anti-Fdx polyclonal serum (mouse); 1:500 (provided by I. Hrdý, Department of Parasitology, Charles University in Prague)

  anti-FOO polyclonal serum (rabbit); 1:500 (provided by J. Lukeš, Institute of parasitology Academy of Sciences of the CZ, České Budějovice)

  anti-HaHa monoclonal antibody (mouse IgG); 1:2000 (provided by Felix Bastida, LA, USA)

  anti-5xHis monoclonal antibody (mouse IgG); 1: 1000 (QiaGen)

  anti-ME polyclonal serum (rabbit); 1:1000 [58]

  anti-PFO monoclonal antibody (mouse IgG); 1:250 (provided by G. Brugerolle, Université Blaise – Pascal de Clermont – Ferrand, France)

  anti-*strep*-tag II antibody (mouse IgG); 1:4000 (IBA)

  anti-STK polyclonal serum (rabbit IgG); 1:5000 (provided by P. Johnson, LA, USA)

  Secondary antibodies:

  A. Western blot analyses:

  anti-rabbit IgG antibody conjugated with AP (ICN/CAPPEL); 1:2000

  anti-mouse IgG antibody conjugated with AP (ICN/CAPPEL); 1:2000

  B. Immunofluorescence:

  anti mouse IgG labeled with ALEXA FLUOR 488 (Molecular Probec, #A21202), 1:1000

  anti rabbit IgG labeled with ALEXA FLUOR 549 (Molecular Probec, #A21207), 1:1000

- Blocking solution – Immunofluorescence:

  PBS solution

  BSA.....0.25 %

  gelatine.....0.25 %

  (Gelatin from porcine skin Type A, Sigma, # G2500)

- Blocking solution – Western Blot:

  PBS

  non-fat dry milk (Laktino).....10 %

  Tween 20 (MP Biomedicals, Inc).....0.25 %

- Blocking solution – Western Blot with *Strep*-tag antibody:

  PBS

  BSA.....3 %

  Tween 20 (MP Biomedicals, Inc)..... 0.5 %

- Blotting buffer:

  10 x concentrated SDS buffer.....100 ml

  methanol.....200 ml

  distilled water.....700 ml

- Buffers for isolation of rec. His-FOO:

  Lysis buffer (pH 8.0):

  NaH_2PO_4.....50 mM

  NaCl.....300 mM

  imidazole.....10 mM

  Wash buffer (pH 8.0):

  Na H_2PO_4.....50 mM

  NaCl.....300 mM

  imidazole.....20 mM

  Elution buffer (pH 8.0):

  NaH_2PO_4.....50 mM

  NaCl.....300 mM

Imidazole.....400 mM

- Buffers for isolation of TvFdx:

  Buffer A (pH 8.0):

  TRIS.....50 mM

  Buffer B (pH 8.0):

  TRIS.....50 mM

  NaCl.....1 M

- Coenzyme A (CoA) ~8,68 mM:

  CoA.....16 mg

  Glutathion.....192 mg

  0.1 M phosphate buffer, pH 6.8.....2 ml

  incubate for 1 hour at room temperature, then add 0.4 ml of 0.5 M EDTA, pH 6.8

- Coomasie Brilliant Blue R-250 (CBB):

  Coomasie Brilliant Blue R 250.....250 mg

  H_2O.....225 ml

  methanol.....225 ml

  glacial acetic acid.....50 ml

- Destaining solution:

  methanol.....50 ml

  glacial acetic acid.....20 ml

  distil. H_2O.....65 ml

- dichlorophenolindophenol (DCIP).....50 mM

- DNAse I (Promega).....1000U/ml

- Isolation medium (IM):

  sucrose.....77 g

  KH_2PO_4.....1.36 g

Tris.....2.42 g

KCl.....1.49 g

$MgCl_2.6H_2O$ (0,5 M).....10 ml

EDTA (0,1 M).....10 ml

Milli Q H_2O.....add to 1000 ml

pH adjusted to 7.2 with HCl

- LB medium:

  YE (yeast extract).....5 g

  Bacto-Tryptone.....10 g

  NaCl.....10 g

  distilled water.....to 500 ml

  (agar 6 g/ 500 ml LB) – only for plates with stiff LB medium preparation

- Leupeptin.....5 mg/ml

- Lysozyme..... 10 mg/ml

- Lowry reagents:

  A. Na_2CO_3.....2 %

     NaOH.....0.1 M

  B. $CuSO_4$ .....0.25 g

     NaK Tartrate.....0.5 g

     before blending dissolve both salts well, pour together and fill up with distilled H_2O to volume 50 ml

  C. Folin-Ciocalteu's Reagent (FC), dilute the new stock with distilled water (1:2)

  D. 1 ml of solution B and 50 ml of solution A, mix fresh

     BSA Standard - 1 mg/ ml

- Merlin I:

  glucose.....50 mM

  Tris HCl, pH 8.0.....25 mM

EDTA, pH 8.0.....10 mM

RNAse A without DNAse activity (Fermentas).....10 µg/ml

Store in the fridge.

- Merlin II:

  NaOH.....0.2 M

  SDS.....1 %

- Merlin III (3 M potassium acetate, pH 5.5):

  solid potassium acetate.....61.35 g

  glacial acetic acid.....35.7 ml

  distilled H_2O.....to 500 ml

- MerlinMax binding buffer (Merlin IV):

  To make 200 ml:

  Guanidine hydrochloride.....133.68g

  Merlin III buffer .....66.67 ml

  distilled H_2O.....40 ml

  Stir in a very clean 250 ml glass beaker with gentle heating. Adjust pH to 5.5.

- Merlin resin slurry:

  Diatomaceous Earth (Sigma, #D5384).....30 g

  Merlin IV solution.....200 ml

  (stir and store at RT until use)

- Merlin V (column wash solution):

  NaCl.....200 mM

  Tris HCl, pH 7.5..... 20 mM

  EDTA.....5 mM

  ethanol.....50 %

- methylviologen (N,N'-dimethyl-4,4'-bipyridinium, MV) (Sigma):

  400mM

- PBS:

  NaCl.....8 g

  KCl.....0.2 g

  $Na_2HPO_4.12H_2O$.....1.53 g

  KH_2PO_4.....0.2 g

  distilled H_2O.....to1000 ml

  pH adjusted to 7.4

- Physiologic solution:

  NaCl.....9 g

  distilled H_2O.....1000 ml

- Ponceau S staining solution:

  Ponceau S.....0.5 %

  glacial acetic acid.....1 %

- RNAse A (Fermentas).....10 mg/ml

- SDS buffer – 10x concentrated:

  Trizma Base (Sigma)..... 30.3 g

  glycine.....144 g

  SDS..... 10 g.

  H_2O.....1000 ml

  store at RT

- SOC medium:

  caseine hydrolysate.....2 %

  yeast extract.....0.5 %

  NaCl.....10 mM

  KCl.....2.5 mM

  $MgCl_2$.....10 mM

  $MgSO_4$.....10 mM

glucose.....20 mM

pH 7.0 (sterilized by autoclave 120°C, 20 min.)

- ST buffer

  Sucrose.....250 mM

  TRIS.....10 mM

  KCl.....0.5 mM

  pH =7.2 adjusted with HCl

- 2 x ST buffer

  sucrose.....500 mM

  TRIS.....20 mM

  KCl.....1 mM

  pH =7.2 adjusted with HCl

- Tosyl-lysin-chlormetylketon (TLCK).....25 mg/ml

- TYM medium:

  Tryptone (Oxoid).....20.0 g

  Yeast extract (Oxoid).....10.0 g

  maltose (Sigma).....5.0 g

  L-cystein.....1.0 g

  ascorbic acid (Sigma)..... 0.2 g

  KH_2PO_4 (Fluka).....0.8 g

  K_2HPO_4 (Fluka).....0.8 g

  agar (Oxoid, L28).....0.5 g

  distilled water.....to 900 ml

  pH was adjusted with KOH to 6.2. Medium was sterilized in autoclave (120°C, 20 minutes) and after sterilization inactivated horse serum was added to 10% concentration (Horse serum inactivation: 30min., 56°C).

4.2 Organisms

4.2.1 *Trichomonas vaginalis*

T. vaginalis strain T1 (TvT1) (J.-H. Tai, Institute of Biomedical Sciences, Taipei, Taiwan) was used for transfection and activity determinations.

T. vaginalis metronidazole-resistant strain TV 10-02 MR100 (highly-resistant, laboratory derived strain) [67] was used for Western blot and immunodetection of FOO in metronidazole-resistant trichomonads.

4.2.1.1 Cultivation of *T. vaginalis* and preparation of cultures for experiments

The strains of *T. vaginalis* were routinely grown axenically in modified Diamond's medium TYM [110] with agar at 37°C. Transfers to fresh medium were made every 24 or 48 hours, depending on the size of inoculum.

Large-volume cultures were grown in 1 l bottles (900 ml TYM medium without agar, 100 ml inoculum) and harvested after 24 hours of growth (late logarithmic phase).

4.2.2 *Escherichia coli*

The XL1 blue *E. coli* cells were used for transformation and cloning work. For purification of recombinant *T. vaginalis* His-FOO, *E.coli* M15 strain (I. Hrdý, Department of Parasitology) was used.

For purification of recombinant *T. vaginalis* ferredoxin (TvFdx), BL 21 *E. coli* cells transformed with *pET*-3a plasmid containing ferredoxin gene were used.

4.3 Basic laboratory techniques

4.3.1 Analysis of DNA samples by electrophoresis in agarose gel

The samples (after PCR, restriction cleavage, isolation of plasmids) were analysed in 1% agarose gel with ethidium bromide by horizontal electrophoresis.

The sample was mixed with 6 x concentrated sample buffer (Fermentas) and then loaded on electrophoresis.

Electrophoresis was performed under constant voltage 80V. The size of DNA fragments was determined by comparison with standard 1kb DNA Ladder (Fermentas). DNA stained with ethidium bromide was visualised by UV transilluminator.

4.3.2 DNA extraction from agarose gel

After interpretation of electrophoresis, the required DNA band was cut out from the gel using sterile scalpel. The DNA was purified using QIAquick Gel Extraction Kit (250) (Qiagen).

4.3.3 Transformation of E. coli XL1 blue

The *E. coli* XL1 blue strain was transformed with thermal shock (30 seconds at 42°C). Subsequently, the cells were incubated on shaker at 225 RPM/ 37°C/ 1hr after addition of preheated SOC medium (37°C). Then the germs were seeded on agar plates with LB medium and ampicilin (100μg/ml) and incubated with plate bottom up for 24 hours at 37°C.

4.3.4 SDS – PAGE

Proteins were separated according to molecular mass by standard vertical electrophoresis in 12% polyacrylamide gel with sodium dodecyl sulfate (SDS –PAGE) [111]. Before loading on electrophoresis (Mini Protean II; BioRad) the samples were mixed with SDS –PAGE sample buffer and then boiled in water bath at 100°C for 5 minutes.
For molecular weight determination standard Dalton Mark VII-L (Sigma) was used. After electrophoresis the proteins were visualized by staining the gel with Coomassie Brillliant Blue R-250 (CBB) solution.

4.3.5 Western blotting

Protein transfer on nitrocellulose membrane (Protran, Schleicher & Schuell):

Semi-dry blotting apparatus (Biometra) and standard blotting unit (BioRad) were used.
Transfer conditions:
Transfers with Fast Blot B-31 (Biometra): constant current 1.5 mA x cm^2 of membrane; 1 hour 15 min

Transfers with Mini Trans Blot Cell (BioRad): constant current 350 mA; 1 hour

The proteins on membrane were stained with Ponceau S; the molecular weight standards were marked, membrane was destained in water.

4.3.6 Immunodetection of proteins

Procedure:

1. Block the nitrocellulose membrane in blocking solution (paragraph 4.1) at room temperature (RT) for 2 hours or overnight at 4°C.
2. Incubate the membrane for 1 hour with primary antibody in blocking solution at RT.
3. Rinse the membrane 3 x 15 min. in blocking solution.
4. Incubate the membrane for 1 hour with secondary antibody in blocking solution at RT.
5. Rinse the membrane 2 x 15 min. in blocking solution and 1x 15 min in PBS.
6. The proteins on membrane are detectable by chemiluminiscence (ECL) or staining reaction with alkaline phosphatase (AP).

4.3.7 Immunofluorescence

4.3.7.1. Preparation of slides

Procedure:

1. Incubate cleansed slides 20 min in 1M HCl.
2. Rinse the slides with slow flow of water for 30 minutes.
3. Wet the slides in distilled water 2 x 5 min.
4. Incubate in acetone 30min.
5. Dry.
6. Sink into 2% 3-aminopropyltriethoxylan (Sigma) solution in acetone for 30 min.
7. Rinse the slides with slow flow water for 30 min.
8. Rinse 2 x 5 min in distilled water.
9. Bake at 100°C for 60 min.

4.3.7.2. Preparation of mounts

Procedure:

1. Let fall a drop of cell suspension on the slide.
2. Incubate at 37 °C 15 min at wet cell, in thermostat.

3. Let the mount dry.
4. Fix the cells in -20°C cold methanol 5 min, then permeabilize in –20°C cold acetone.
5. Repeat the step 3.
6. Block the mounts for 1 hour in blocking solution in dark and wet.
7. Incubate mounts with diluted primary antibody in blocking solution (paragraph 4.1) for 1 hour in dark and wet.
8. Rinse the mount 3 x 5 min in PBS.
9. Incubate with secondary antibody for 1 hour in dark and wet.
10. Rinse 3 x 5 min in PBS.
11. Assemble the mounts in potting medium Vectashield with DAPI (Vector laboratories).

4.3.8 MERLIN MAXI – PREPS

Reagents (section 4.1):

- Merlin I
- Merlin II
- Merlin III
- MerlinMax binding buffer (Merlin IV)
- Merlin resin slurry
- Merlin V (column wash solution)

All solutions have to be sterilized by autoclave.

Procedure:

1. Spin 1 liter overnight bacterial culture at 3600 x g/ 10min./ 4°C (Janetzki K70 centrifuge). Decant supernatant and resuspend the bacterial pellet in 10 ml of solution Merlin I and transfer it into 50 ml plastic tube (Falcon).
2. Add 10 ml of solution Merlin II, swirl and keep at RT for 5 min., exactly.
3. Add 10 ml of solution Merlin III, mix by shaking and keep on ice for 30 min.
4. Centrifuge 50 000 x g/ 20 min./ 4°C (Beckman, rotor Ti 50.2). Pour off supernatant into a 50 ml sterile Falcon tube.
5. Add 0.6 volumes of isopropanol. Shake well to mix and spin at 8960 x g/ 1 hour/ 25°C (Hettich 32R centrifuge). (In this step it is possible to let DNA precipitate in isopropanol over night at -20°C)

6. Decant supernatant and briefly allow the DNA pellet to dry by inversion of the bottle over a paper towel.
7. Dissolve the DNA pellet in 2 ml of HPLC water.
8. Add 15 ml of MerlinMax binding buffer and 5 ml of MerlinMAX Resin Slurry (before use, shake the slurry vigorously for a few minutes to resuspend!).
9. Mix with gentle shaking on a rocker platform for 20 min. at RT.
10. Load the mixture resin slurry-DNA onto column (Econo-Pac Disposable Chromatography columns, BioRad) connected to a vacuum line under constant suction. Allow the resin to set under vacuum, after all the buffer has been sucked trough and the resin bed is dry, top off the column with Merlin V solution (about 20 ml). Allow washing of the column by suction. Add another 20 ml of Merlin V. After the bed is dry continue suction for at least 5 min. to remove all traces of ethanol from the column.
11. Disconnect the vacuum line and cut the column in about half, add 1.5 ml of pre- heated 80°C HPLC water. Place the column into 50 ml tube (Falcon) and spin at 8960 x g/ 5 min./ 25°C (Hettich 32R centrifuge).
12. Repeat elution by H_2O three times.
13. Precipitate plasmids with 2.5 multiple of their volume by ethanol.
14. Spin at 8960 x g/ 30 min./ 25°C (Hettich 32R centrifuge), allow the pellets dry a then dissolve in 0.5 ml HPLC water.
15. Measure optical density (OD) of isolated DNA at wavelength 260 nm and 280 nm and from acquired readings calculate the concentration of plasmid DNA in the sample.

OD DNA was measured spectrophotometrically using UV-1601 UV-visible Spectrophotometer (Shimadzu).

Concentration of DNA was calculated using the formula

$$c\ (\mu g/ml) = 50 \cdot OD_{260} \cdot \text{sample dilution}$$

$$c\ (\mu g/\mu l) = c\ (\mu g/ml)/\ 1000$$

4.3.9 Harvesting of the cells and isolation of hydrogenosomes

Reagents (4.1):

- Physiologic solution
- ST buffer
- 2 x ST buffer
- stock solutions of protease inhibitors

Procedure:

1. Spin the late-logarithmic culture at 2000 x g/ 15min./ 4°C (Janetzki K 70 centrifuge). Remove the supernatant by aspiration.
2. Resuspend the pellet in physiological solution and spin again. Remove the supernatant by aspiration.
3. Repeat step 2.
4. Resuspend the pellet in about 50 ml of ST buffer and transfer the suspension into 50 ml screw cap Falcon tube. Spin the cells 1000 x g/ 5 min./4°C (Hettich 32R centrifuge) and remove supernatant.
5. Resuspend the cells in 20 ml ST buffer and add inhibitors of proteases to final concentration 10μg/ml leupeptin and 50μg/ml TLCK.
6. Since this step keep the suspension on ice and gently bubble the cells with nitrogen for 5 minutes.
7. Homogenize by sonicator Vibra Cell 2-3 times at amplitude 40 – 50, 1 sec pulses, in one minute intervals. After every interval shake suspension and check the cells microscopically. Sonicate until only few cells remain unbroken.
8. Spin the homogenate at 600 x g/ 10 min./ 4°C (Hettich 32R centrifuge). Sediment contains unbroken cells and nuclei.
9. Spin the supernatant at 22 820 x g/ 30 min./ 4°C (Beckman Optima L centrifuge, rotor Ti-50.2). The pellet forms LGF (Large Granule Fraction) enriched in cell organelles, lysosomes and hydrogenosomes. The supernatant contains cytoplasm.
10. Rinse LGF twice in 1 ml of ST buffer and spin at 10 000 x g/ 10 min/ 4°C. Suck the white lysosomes carefully off the top of brown hydrogenosomal pellet. Resuspend in 250 μl ST buffer, add inhibitors of proteases TLCK (50μg/ml) and Leupeptin (10μg/ml).

11. Before processing keep cytoplasm, homogenate and hydrogenosomes on ice, then store at -70°C.

Hydrogenosomes of higher purity can be obtained by isopycnic centrifugation on self-formed Percoll gradient:

1. Fill four 36.2 ml Opti Seal tubes for VC-53 vertical Beckman rotor with 45% (final) Percoll solution:

   2 x ST buffer.....18 ml

   Percoll.....16.2 ml

   Water.....1.8 ml

   (TLCK).....72 µl

   (Leupeptin).....72 µl

2. Invert the tubes several times to mix the solution. Remove about 3 ml of Percoll solution from the tube. Transfer the suspension of LGF obtained in step 9 of harvesting cells into the tube and then refill the tube completely with Percoll solution. Insert the stopper and invert several times.
3. Spin in Beckman VC-53 rotor at 67 000 x g for 30 min. without brake. Hydrogenosomes are the lower, darker band, lysosomes are less dense and white (Fig. 9).
4. Remove the hydrogenosomes using pipette, dilute about 10 fold with ST buffer and wash them in Beckman Ti-50.2 rotor at 22 820 x g for 10 min.
5. Add protease inhibitors and store at -70°C.

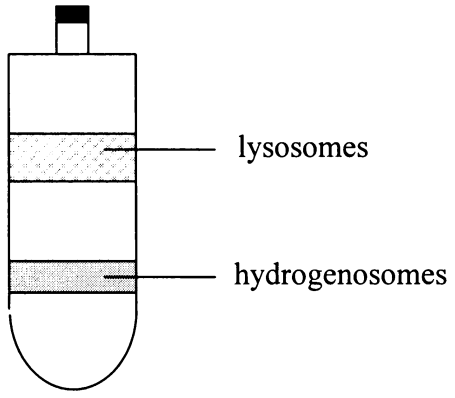


Figure 9. Distribution of hydrogenosomes and lysosomes after isopycnic centrifugation on 45% Percoll gradient.

4.3.10 Transformation of trichomonads by electroporation

Procedure:

1. Spin 1 litre culture of *T. vaginalis* strain T1 in sterile way using 50 ml plastic tubes (Falcon) at 1000 x g/ 15 min./ 4°C (Hettich 32R centrifuge).
2. Weigh the cell pellet and add 0.5 ml TYM medium, without agar, with additional penicillin (1000 U/ml) and amikacin (250 U/ml) per each gram of pellet.
3. Draw the suspension trough the 23G needle four times.
4. Pipette 300 µl of cells and 50 µg of recombinant plasmid (volume of DNA should be under 30 µl) into 0.4 cm cuvette (4MM Gene Pulser Cuvette, BioRad) on ice.
5. Electroporate at time constant 175 ms at 350 V, or at 350 V and 975 µF (electroporator GenePulser Xcell, BioRad).
6. Add preheated TYM 6.2 (without agar, with addition of penicillin (1000 U/ml) and amikacin (250 U/ml) to the cells in cuvette and by glass pasteur pipette transfer the cells into 50 ml tubes with the same TYM 6.2 medium. Incubate at 37°C.
7. After 4 hours add to trichomonads geneticin (200 µl/ml) for positive selection of transformants.
8. After 24 hours, when the culture is grown well, spin it at 990 x g/ 15 min./ 4°C (Hettich 32R centrifuge) and suck off upper 45 ml of medium.
9. Resuspend the pellet in fresh TYM 6.2 medium without agar, with penicillin (1000 U/ml), amikacin (250 U/ml) and geneticin (200 µg/ml).
10. After 3 days it's generally needed to transfer the cells into 10 ml tubes with TYM 6.2 medium (with agar) and continue with positive selection of transformants by geneticin. After selection of positive transformants, it's enough to keep the cells at concentration 100 µg/ml of geneticin.

4.3.11 Isolation of recombinant T. vaginalis ferredoxin

T. vaginalis ferredoxin (TvFdx) was purified from 1.5 liters of BL 21 *E. coli* cells transformed with *pET*-3a plasmid containing ferredoxin gene (gift of Juris P. Germanas, University of Houston, Texas).

- Buffers for isolation of TvFdx (paragraph 4.1)

Procedure:

1. Prepare 50 ml of LB with final concentration 100 μg/ml of kanamycin, 30 μg/ml of chloramfenicol and inoculate the startup culture. Incubate at 37°C overnight on shaker.
2. In the morning, add antibiotics to 1.5 l LB with the same final concentration of antibiotics (100 μg/ml of kanamycin, 30 μg/ml of chloramfenicol).
3. Add 50 ml of startup culture to 1.5 l LB in the flask. Incubate at 150 RPM/ 37°C/ L-med on shaker.
4. Measure the OD_{600} periodically. Induce the culture when the OD_{600} reaches between 0.6 – 0.8. To induce, add 71.5 mg IPTG to make 0.2 mM final concentration and 3 ml of 100 mM ammonium ferrous sulphate.
5. Incubate at least 6 hours at 150 RPM/ 37°C/ L-med.
6. Let the culture stay in the coolroom overnight, because of better ferredoxin clustering.
7. Next day spin the culture at 2000 x g/ 25min./ 10°C (Janetzki K 70 centrifuge).
8. Resuspend the pellet in physiologic solution and transfer into 50 ml plastic tube.
9. Spin at 6000 x g / 15 min./ 10°C (Hettich 32R centrifuge) and remove the supernatant by decanting.
10. Resuspend the cells in buffer A (section 4.1), spin as in the step 9 (in this step is possible to disconnect the process and store the cells at -70°C).
11. Add buffer A to final volume 20 ml and 1 mg/ml of lysozyme. Keep the cells on ice.
12. Freeze the cell suspension in liquid nitrogen and consequently thaw in water bath at 37°C. Repeat three times.
13. Add 15 μl of TLCK and Leupeptin, 50 μl DNAse, 30 μl RNAse (section 4.1) and incubate for 30 min. on ice.
14. Homogenize the cells by Vibra Cell sonicator 4 times at amplitude 40 – 60, 1 sec pulses, in 1 minute intervals.
15. Centrifuge the homogenate at 9 000 x g/ 20 min./ 4°C (Hettich 32R centrifuge).
16. Transfer the supernatant and centrifuge it at 200 000 x g/ 30 min./ 4°C (Beckman Optima L, rotor Ti 50.2).
17. Transfer the supernatant carefully into clean tube and dilute it up to 25 ml with buffer A.

Recombinant ferredoxin contained in the bacterial cytoplasm was purified by two steps of liquid chromatography using BioLogic HR medium pressure system (BioRad). First purification step was done by anion exchange chromatography on DEAE-Sephacel column (XK 16 Pharmacia) equilibrated with buffer A with gradient of 0 –1 M NaCl and

flow rate of 2 ml/min. Fractions containing ferredoxin were concentrated on Amicon Ultra concentrators (Millipore, permeability to 10 kDa) by centrifugation at 4000 x g/ 15 min./ 10°C (Hettich 32R centrifuge). Ferredoxin was further purified by isocratic elution on Superose 12 (K26 Pharmacia column) with flow rate of 2ml/min with buffer A. Ferredoxin fractions were again concentrated and stored at -70°C.

4.3.12 Cultivation and isolation of recombinant FOO

Recombinant *T. vaginalis* FOO protein was isolated from M15 *E. coli* cells transformed with pQE 30 plasmid containing FOO gene with N-terminal 6xHis-tag.

- Buffers for isolation of FOO (paragraph 4.1)

Procedure:

1. Prepare 50 ml of LB medium with final concentration 100 μg/ml of ampicilin, 25 μg/ml of kanamycin and inoculate with M15 *E. coli* cells containing FOO gene in plasmid. Incubate the startup culture at 37°C overnight on shaker.
2. Next morning, add the startup culture into the flask with 1.5 l LB with the same concentrations of antibiotics (kanamycin - 25 μg/ml; ampicilin - 100 μg/ml) and incubate at 150 RPM/ 37°C/ L-med on shaker.
3. Measure the OD_{600} periodically. Induce the culture when the OD_{600} reaches between 0.5 – 0.8. To induce, add 178.72 mg IPTG to make 0.5 mM final concentration, 3 ml of 100 mM ammonium ferrous sulphate sterile stock and 1.5 ml of 100mM FMN sterile stock.
4. Decrease the temperature to 28° and shake 6 hours at least. (After this step it's possible to let the culture stay in the coolroom overnight.)
5. Spin the culture at 2000 x g/ 20min./ 10°C (Janetzki K 70 centrifuge).
6. Resuspend the pellet in physiologic solution and transfer into 50 ml plastic tube. Spin at 6000 x g/ 10 min./ 4°C (Hettich 32R centrifuge).
7. Wash the cells with lysis buffer (section 4.1) and spin at 6000 x g/ 10 min./ 4°C (Hettich 32R centrifuge).
8. Resuspend the sediment in 10ml of lysis buffer (section 4.1), add glycerol to 10% final concentration, 7 μl of TLCK and 7μl of Leupeptin (section 4.1). Iin this step is possible to disconnect the process and store the cells at -70°C.

9. Keep the cells on ice and add another 50 µl of TLCK and Leupeptin.
10. Dilute the cells with lysis buffer to 15ml volume and add 1mg/ml of lysozyme.
11. Freeze the cell suspension in liquid nitrogen and consequently thaw in water bath at 37°C. Repeat three times.
12. Add 15 µl of RNAse and 40 µl DNAse. Incubate for 30 min. on ice.
13. Sonicate the cells (Vibra Cell sonicator) at amplitude 40-60, 1 sec. pulses in 1 min. intervals 3 times.
14. Spin the homogenate at 9000 x g/ 20min./ 4°C (Hettich 32R centrifuge).
15. Spin the supernatant at 250 000 x g/ 30 min./ 4°C (Beckman Optima L, rotor Ti 50.2)
16. Take the supernatant and purify the rec. His-FOO by affinity chromatography.

The protein sample was injected into Ni-NTA column (Qiagen) and eluted with stepwise gradient of 20 and 400 mM imidazole (elution buffer) at the flow rate of 1 ml/min. FOO-containing fractions were concentrated (Amicon Ultra Concentrator, Millipore) and analysed by SDS-PAGE.

4.3.13 Protein determination

Protein concentrations were determined with Folin reagent by the Lowry method [112] using BSA as protein standard.

- Lowry reagents (section 4.1)

Procedure:

[Run duplicate determination for all samples.]

1. Pipette defined volume of samples (5 – 100 µg of protein) into disposable test tubes.
2. To obtain standard curve pipette 0, 5, 25, 50, 75, 100 µl of BSA standard to test tubes.
3. Fill up all samples and standards to 0.5 ml with distilled water.
4. Add 2.5 ml of solution D to each test tube and incubate for 15 minutes at RT.
5. Add 0.25 ml of solution C to each tube and vortex each tube immediately.
6. Incubate at RT for 30 - 35 minutes.
7. Determine absorbance of each sample at 670 nm.
8. Plot absorbance vs µg protein to obtain standard curve.

9. Set up triplicate assays for all "unknowns" and read concentration of proteins from standard curve.

4.4 Characterisation of FOO cofactor by TLC

For determination of FOO cofactor, M15 *E. coli* cells producing recombinant FOO protein were grown without addition of FMN. After purification on Ni-NTA column, the fractions containing FOO were concentrated using ultr filters Amicon Ultra Concentrator (Millipore) at 12000 x g/ 4°C to the volume of about 200 µl, resuspend in 2 ml of distilled water and again concentrated to the volume of less then 100 µl. This concentrated sample was denatured at 95°C for 20 minutes and centrifuged at 13000 x g/ 20 min./ 4°C. The supernatant was consequently concentrated on heatblock at 60°C under the nitrogen flow to the volume of about 30 µl. The sample concentrated this way was applied on TLC plate with alumina_adsorbent (HPTLC-Alufolien, MERCK). As standards 0.6 mM solutions of FAD and FMN were used. The mobile phase consisted of n-butanol : acetic acid : water (6:2:4 volume). The spots were detected under the UV radiation and retention factor (Rf) was defined. The retention factor is defined as the distance traveled by the compound divided by the distance traveled by the solvent:

$$Rf = \frac{\text{distance traveled by the compound}}{\text{distance traveled by the solvent front}}$$

4.5 Determination of native molecular weight of FOO

The native molecular weight of ferredoxin:oxygen oxidoreductase was defined by gel filtration chromatography. The column with SUPERDEX 75 (XK 16 column, Pharmacia) was equilibrated with 400 mM imidazole, 50 mM natrium phosphate buffer, pH 8.0.

The recombinant FOO or the hydrogenosomal extract of FOO-overexpressing trichomonads (TvFOOWT) was run on the column using the flow rate of 1ml/min. The native molecular weight of enzyme was calculated from the calibration curve determined by running the standards (158 kDa, 44kDa, 17 kDa and 1.35 kDa) under the same conditions (Table 1.).

Table 1. Molecular weight values and elution volumes of protein standards used for calibration curve construction.

Relative molecular weight (MW)/ Da	Elution volume/ ml
158 000	26.90
44 000	33.20
17 000	40.80
1 350	57.40

4.6 Spectrophotometric determination of enzymatic activities

Activities of several hydrogenosomal enzymes were measured spectrophotometrically at relevant wavelengths as the rate of extinction change in the reaction cuvette (light pass 1 cm). The measurements were carried out on UV-1601 UV-visible Spectrophotometer (Shimadzu). One unit of enzymatic activity (U) was defined as amount of protein catalyzing consumption of one micromole of substrate or formation formation of one micromole of product per minute.

Specific activity (SA) of given enzyme was calculated using following formula:

$$\text{Specific activity (SA)} = \Delta A.V / \varepsilon_{\lambda}.p.v \ [\mu mol/min/mg \text{ protein}]$$

ΔA.....extinction change in 1 minute

V.....volume of reaction mixture in cuvette

p.....amount of protein in measured sample [mg/ml]

v.....volume of protein sample

ε_{λ}.....molar extinction coefficient at relevant wavelength

λ.....wavelength [nm]

ε_{340} (NADPH; NADH) = 6.22 $mM^{-1}.cm^{-1}$

ε_{600} (MV) = 6.3 $mM^{-1}.cm^{-1}$

ε_{600} (DCIP) = 21 $mM^{-1}.cm^{-1}$

All activity determinations were done at least in triplicates. Mean values with standard deviations are presented in all graphs.

4.6.1 Determination of hydrogenase activity

Solutions and reagents:

400 mM Methylviologen (MV)

Mercaptoethanol (MerOH)

IM

The anaerobic cuvette with two arms was filled as follows:

- 1. side arm: 3 granules of palladium closed with cotton wool
- 2. side arm: 5-20 µl of sample (hydrogenosomes) washed down with 20 µl IM
- main space of cuvette:

IM.....2 ml

MV.....50 µl

MerOH.....20 µl

Filled cuvette was closed and gently bubbled with oxygen-free H_2 for 10 minutes. Thereafter the activity of hydrogenase was measured at the wavelength λ = 600nm.

4.6.2 Determination of pyruvate: ferredoxin oxidoreductase (PFO) activity

Solutions and reagents:

0,1 M phosphate buffer (KH_2PO_4/KOH), pH 7.4

~714 mM pyruvate (always fresh)

~400 mM MV

~8.68 mM CoA

MerOH

The anaerobic cuvette was filled as follows:

- side arm: 1% TX 100.....20µl

  Sample.....10 - 100µl
- main space of cuvette:

  0,1 M phosphate buffer...2 ml

  MV.....50µl

  CoA.....50µl

  MerOH.....20µl

pyruvate.....20µl

The cuvette was closed and bubbled with oxygen-free N_2 for 5 minutes before mixing reagents and measuring the activity at wavelength λ = 600 nm.

4.6.3 Determination of Complex I activity

Solutions and reagents:

100 mM TRIS, 50 mM KCl, pH 8.0

~337mM NADH

50 mM DCIP

- Reaction mixture:

100 mM TRIS, 50 mM KCl..2 ml

NADH.....20 µl

DCIP.....3 µl

10% TX 100.....5 µl

The reaction was started by addition of hydrogenosomal sample. The activity was measured at λ = 600 nm.

4.6.4 Determination of malic enzyme (ME) activity

Solutions and reagents:

100mM β-NAD

0,5 M L-malate

- Reaction mixture:

IM.....2 ml

NAD.....20 µl

L-malate.....40 µl

10% TX 100.....5 µl

The reaction was started by adding of hydrogenosomal sample. The activity was measured at λ = 340 nm.

4.7 Respiration

Respiration experiments were performed using oxygen-monitoring Clark-type electrode (Strathkelvin Instruments, Scotland, UK). The electrode was calibrated with oxygen - saturated water and sodium dithionite solution (zero oxygen) at 25°C.

Respiration rate determinations (expressed as μmol O_2 consumed per mg of protein per minute) were done at least in triplicates. Mean values with standard deviations are presented in all graphs.

4.7.1 Respiration of hydrogenosomes

Hydrogenosomal respiration was determined using oxygen monitoring Clark-type electrode at constant temperature 25°C. Both NADH-dependent and pyruvate-dependent respiration was determined this way.

Reaction mixtures:

- NADH-dependent respiration

  IM.....500 μl

  NADH (20mM).....10 μl

  10% TX 100.....5 μl

  hydrogenosomes.....2.5 – 10 μl

  (Tv Fdx.....5 – 10 μl)

- Pyruvate-dependent respiration

  IM.....500 μl

  ADP (50 mM).....10 μl

  CoA (~8.68 mM).....10 μl

  pyruvate (500 mM).....10 μl

  succinate (500 mM).....2 μl

  10% TX 100.....5 μl

  hydrogenosomes.....2.5 – 10 μl

  (Tv Fdx.....5 – 10 μl)

4.7.2 Respiration of recombinant His-FOO

Recombinant His-FOO protein was purified from M15 *E. coli* cells lysate (paragraph 4.3.12) and used in respiration experiments in isolated system. Experimental setup was as follows:

100mM Tris, 50mM KCl, pH 8.0.....0.5 ml
rec. TvFdx.....15 µl (~8mg/ml)
Complex I.....5 µl (~0.12 mg/ml)
NADH (20mM).....10 µl
rec. His-FOO.....5 µl (~2.5 mg/ml)

Complex I was purified by L. Bardoňová (Department of Parasitology, Charles University in Prague).

4.8 Preparation of *T. vaginalis* lines overexpressing FOO

4.8.1 Construction of recombinant plasmid MasterNEO

4.8.1.1 PCR amplification of gene encoding FOO protein

As a template for PCR reaction, DNA from *T. vaginalis* DNA library (XLOR cells, lambda ZAP vector; I. Hrdý, Department of Parasitology) was used.

- Primers:

FOO *NdeI* F:	5'-GCTCAT▼ATGCTCAGCACTTCTAGC-3'
FOO Stop *BamHI* R:	5'-GCTGGA▼TCCTTACTTGCACTTCTCGAT-3'
FOO *Acc65I* R:	5'-GCTGGT▼ACCCTTGCACTTCTCGATGGC-3'

F, forward primer; R, reverse primer; shaded box, restrictase recognition sequence;
▼, cleavage position

PCR product amplified with FOO *NdeI* F and FOO Stop *BamHI* R primer containing Stop codon was used to prepare *T. vaginalis* cell lines overexpressing FOO protein without tag (FOO WT).

PCR product obtained by amplification with FOO *NdeI* F and FOO *Acc65I* R after cloning into MasterNEO vector contained 6xHis or HaHa tag (*FOOHis* and *FOOHaHa*).

- reagents (Fermentas):

10 x PCR buffer

10 mM dNTP

5 U/µl Tag DNA polymerase

2.5 U/µl Pfu polymerase

25 mM $MgSO_4$

25 mM $MgCl_2$

10 µM primers

- components of PCR reaction for total volume of 25 µl:

PCR buffer.....2.5 µl

10 mM dNTP.....0.8 µl

$MgSO_4$*.....3 µl

primer F.....1 µl

primer R.....1 µl

DNA.....1 µl

Tag poly*.....0.2 µl

Pfu poly.....0.2 µl

sterile H_2O.....5.3 µl

*Verifying of positive colonies: Pfu polymerase is replaced by Tag polymerase and $MgSO_4$ by $MgCl_2$

- PCR program:

1. 94°C.....5 min. (denaturation)
2. 94°C.....1 min. (denaturation)
3. 55°C.....30 sec. (annealing)
4. 72°C.....1 min. (extension)
5. GO TO 2., 34 x
6. 72°C.....7 min.
7. 4°C.....for ever

4.8.1.2 TA cloning of PCR product

Amplified DNA was isolated from agarose gel (4.3.1, 4.3.2) and cloned using pGEM®-T Easy Vector System (Promega). Competent XL1 Blue *E. coli* cells were transformed

with heat shock (4.3.3) and grown on agar plates with LB medium containing ampicilin (100μg/ml) (selection of transformed cells carrying plasmid) and X-Gal (selection of cells carrying plasmid with inserted DNA fragment). Positivity of chosen clones was verified by PCR reaction (experimental conditions are described in paragraph 4.8.1.1) and sequence analysis.

Positive cell lines with *FOO* in pGEM®-T easy vector were grown overnight in LB medium with ampicilin (100μg/ml). 1ml of each positive clone was stored in 10 % glycerol at –70°C. Plasmid DNA was isolated by Wizard Plus Miniprep Kit (Promega) from 5 ml of bacterial culture.

4.8.1.3 FOO gene fragment cloning into MasterNEO plasmid

MasterNEO plasmid is expression vector specifically designed to enable stable, selectable transformation of *T. vaginalis* cells (pMasterNEO gently provided by dr. Patricia J. Johnson, Department of Microbiology, Immunology and Molecular Genetics, University of California, USA). Gene for FOO was inserted into this vector via two restriction sites (Fig. 10).

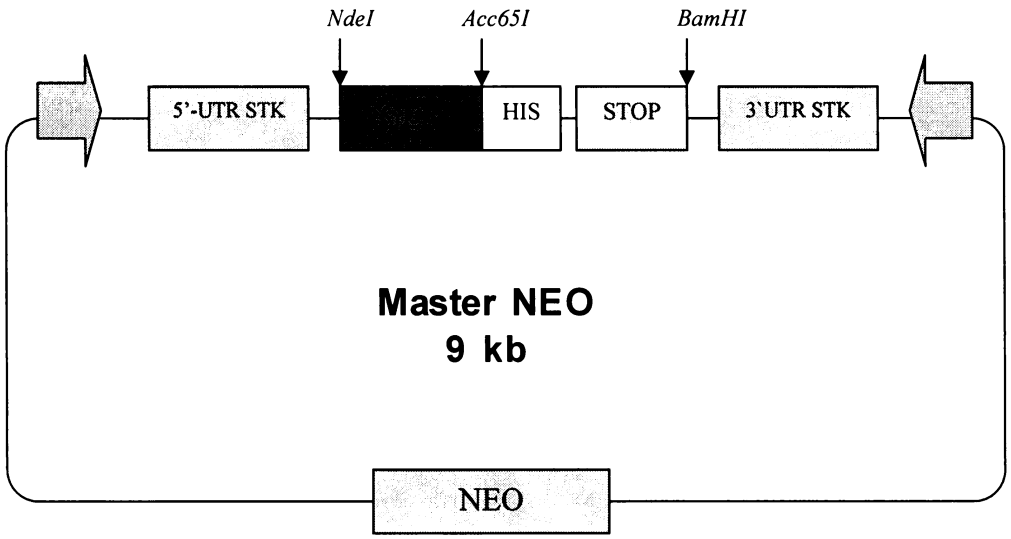


Figure 10. Master NEO expression vector with 6x histidine tag. UTR STK, untranslated regulation area of gene for *T. vaginalis* succinate thiokinase; His, polyhistidine tag; NEO, gene conferring resistance to neomycin.

4.8.1.3.1 Cleavage of FOO from pGEM®-T easy vector and linearization of pMasterNEO

Reaction mixture (Fermentas reagents):

- Tagged constructs

pGEM-(*FOOHis or FOOHaHa*).....0.5 µg
NdeI (10U/ µl).....0.5 µl
Acc65 (10U/ µl).....0.5 µl
1 x ORANGE buffer.....2 µl
deionized sterile water to final volume of 10 µl

pMasterNEO.....0.5 µg
NdeI (10U/ µl).....0.5 µl
Acc65 (10U/ µl).....0.5 µl
1 x ORANGE buffer.....2 µl
deionized sterile water to final volume of 10 µl

- Constructs without tag

pGEM-*FOOWT*.....0.5 µg
NdeI (10U/ µl).....0.5 µl
BamHI (10U/ µl).....0.5 µl
2 x TANGO Y^+ buffer.....4 µl
deionized sterile water to final volume of 10 µl

pMasterNEO.....0.5 µg
NdeI (10U/ µl).....0.5 µl
BamHI (10U/ µl).....0.5 µl
2 x TANGO Y^+ buffer.....4 µl
deionized sterile water to final volume of 10 µl

Reactions run for 4 hour at 37°C.

4.8.1.3.2 Ligation

DNA *FOO* cleaved off pGEM and MasterNEO plasmid linearized with corresponding DNA restrictases were used in ligation by T4 DNA ligase (Fermentas). Reactions run overnight at 16°C. *E. coli* XL1 Blue cells were transformed with recombinant plasmids by heat shock and seeded on agar plates with LB medium and

ampicilin (100 μg/ml). Presence of recombinant plasmids was verified by PCR (experimental conditions are described in paragraph 4.8.1.1) and sequence analysis. MasterNEO plasmid from positive strains was used for transformation of *T. vaginalis* cells (4.3.3).

4.9 Cloning of *FOO* gene into expression vector with *Strep*-tag (TagVag 3)

4.9.1 Construction of T. vaginalis expression vector with Strep-tag

For construction of *T. vaginalis* expression vector with *Strep*-tag, TagVag 2 vector was used. TagVag 2 is a vector designed for transfection of *T. vaginalis* cells (Pavel Doležal, Department of Parasitology, Charles University in Prague). In order to get an expression system enabling easy purification of protein, a short sequence consisting of only 8 amino acids (Trp-Ser-His-Pro-Gln-Phe-Glu-Lys) with tag function was inserted into the TagVag vector. This tag with high affinity to streptavidin was called *Strep*-tag and corresponding vector TagVag 3 (Fig. 11).

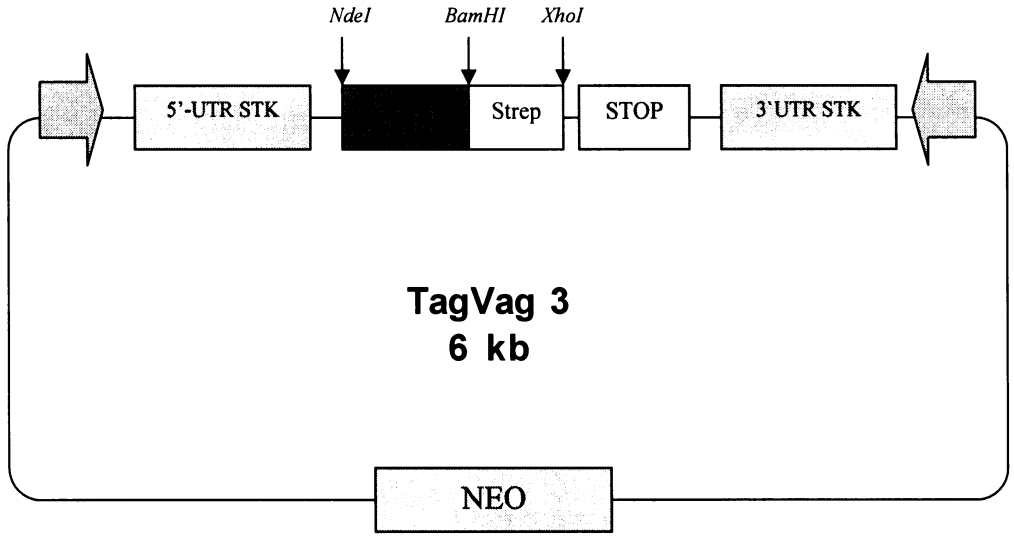


Figure 11. Expression vector TagVag 3. Strep, *Strep*- tag; UTR STK, untranslated regulation area of *T. vaginalis* succinate thiokinase gene; NEO, gene conferring resistance to neomycin.

Two nucleotide sequences, *strep* F and *strep* R, were base paired and inserted into TagVag instead of HaHa tag which was cleaved off.

strep F: 5'-GATCCTGGTCACACCCACAGTTCGAAAAGTGAC-3'

strep R 5'-TCGAGTCACTTTTCGAACTGTGGGTGTGACCAG-3'

4.9.1.1 Base pairing program:

Touchdown PCR program for base pairing was used; 10 µl of *strep* F and *strep* R was used in the reaction.

Touchdown PCR program:

1. 94°C.....5 min.
2. 94°C*.....0:30 min. (annealing)
3. GO TO 1., 42x

(* For every subsequent cycle, the annealing temperature is decreased by 1°C)

4. 4°C.....for ever

Obtained PCR product:

5'- G A T C C T G G T C A C A C C C A C A G T T C G A A A A G T G A C-3'

3'-G A C C A G T G T G G G T G T C A A G C T T T T C A C T G A G C T-5'

BamHI overhang: 5' – GATC

XhoI overhang: 5' – TCGA

4.9.1.2 Linearization of TagVag 2 plasmid:

TagVag 2 plasmid was linearized by restriction endonuclease cleavage with enzymes providing cloning sites appropriate to overhangs of S*trep*-tag insert (All reagents by Fermentas).

pTagVag 2.....1 µg/ 8.2 µl

BamHI (10U/ µl).....0.4 µl

XhoI (10U/ µl).....0.4 µl

1 x GREEN buffer.....1 µl

Restriction run for 3 hours at 37°C and HaHa tag was cleaved off.

Linearized plasmid was isolated from agarose gel (4.3.2) and *strep*-tag was embedded in it.

4.9.1.3 Ligation:

(Reagents by Fermentas)

T4 DNA ligase Buffer .....1 µl

Linearized TagVag 2.....6 µl

Strep-tag .....2 µl

T4 DNA ligase .....1 µl

Reactions run overnight at 16°C.

E. coli XL1 blue cells were transformed with ligation product and seeded on agar plates with LB and ampicilin (100 µg/ml). Positive clones were verified by PCR reaction (paragraph 4.8.1.1) and sequence analysis. Clones carrying *strep*-tag in plasmid (plasmid TagVag 3) were frozen at -70°C with 10 % glycerol.

4.9.2 Insertion of gene for FOO protein into expression vector with Strep-tag

As DNA template for PCR reaction DNA from *T. vaginalis* DNA library (XLOR cells, lambda ZAP vector; I. Hrdý, Department of Parasitology, Charles University in Prague) was used. *FOO* gene was amplified with following at the same conditions as in 4.8.1.1.

- Primers:

FOO *NdeI* F: 5'-GCTCAT▼ATGCTCAGCACTTCTAGC-3'

FOO *BamHI* R: 5'-AGACCGGA▼TCCCTTGCACTTCTCGGAT-3'

F, forward primer; R, reverse primer; shaded boxes are the sequences recognized by restrictase;

▼, cleavage position

PCR product was cloned into pGEM®-T Easy Vector System (Promega) according to the technique described in 4.8.1.2.

4.9.2.1 FOO gene fragment cloning into TagVag 3

FOO gene was cut out from pGEM-T Easy vector by appropriate restrictases and inserted into TagVag 3 plasmid (All buffers and restrictases by Fermentas).

pGEM-(*FOOStrep*).....0.5 µg

NdeI (10U/ µl).....0.5 µl

BamHI (10U/ µl).....0.5 µl

2 x TANGO Y^+ buffer.....4 µl

deionized sterile water to final volume of 10 µl

pTagVag3.....0.5 µg
NdeI (10U/ µl).....0.5 µl
BamHI (10U/ µl).....0.5 µl
2 x TANGO Y^+ buffer.....4 µl
deionized sterile water to final volume of 10 µl

Ligation (reagents by Fermentas):
T4 DNA ligase Buffer .....1 µl
linearized pTagVag3 .....2 µl
FOO.....6 µl
T4 DNA ligase .....1 µl
Reactions run overnight at 16°C.

The competent cells were transformed according to the technique described in 4.3.3. Plasmid DNA from positive clones after sequence analysis were used for transformation of *T. vaginalis* cells. Positivity of transformants was checked by Western blot analysis (chapter 4.3.5).

4.10 The search for hydrogenosomal proteins interacting with FOO

Recombinant FOO fused with 6x His-tag could be easily purified by affinity chromatography on Ni-NTA agarose column with stepwise gradient elution with up to 400 mM imidazole using programmable chromatographic system.

Alternative way of FOO isolation is purification on Ni-NTA agarose in Poly-Prep® chromatography columns (BioRad) using gravity flow. This technique was employed in an attempt to identify possible hydrogenosomal protein(s) (presumably the electron donors) that could bind to His-FOO.

Ni-NTA–purified recombinant His-FOO was incubated with TvT1 hydrogenosomal extracts for 1 hour at RT.

Five parallel experiments (A-E) were done. In each experiment, the hydrogenosomes were treated in different way. A and B parallels were prepared by treating of hydrogenosomes with detergents for 1 hour on ice (A, 2% octylglucoside; B, 1% Triton

X 100), hydrogenosomes in parallel C were destroyed by freezing and thawing (4 cycles) and D, E were controls (D, hydrogenosomes only; E, rec. His-FOO only). The mixture of hydrogenosomes and rec. His-FOO protein was poured directly into the columns. In each column was the same amount of FOO protein (250 µl) and TvT1 hydrogenosomes (300 µl). The final concentration of imidazole in incubation mixtures was brought down to 10 mM by dilution.

Hydrogenosomal extracts were incubated with Ni-NTA purified recombinant His-FOO as follows:

Ni-NTA agarose beads were resuspended in PBS, 1 mM $MgCl_2$, and 1 ml of this matrix was poured to each column.

A. 2% octylglucoside:

   1 ml matrix

   250 µl rec. His-FOO in 400 mM imidazole

   2 ml of hydrogenosomes, 2% octylglucoside

   6.75 ml PBS, 1 mM $MgCl_2$

B. 1% Triton X-100:

   1 ml matrix

   250 µl rec. His-FOO in 400 mM imidazole

   2 ml of hydrogenosomes, 1% TX 100

   6.75 ml PBS 1 mM $MgCl_2$

C. 4x freezing – thawing:

   1 ml matrix

   250 µl rec. His-FOO in 400 mM imidazole

   1 ml of mixture (300 µl TvT1 hydrogenosomes + 700 µl PBS, 1 mM $MgCl_2$)

   7.75 ml PBS 1 mM $MgCl_2$

D. hydrogenosomes only:

   1 ml matrix

   300 µl TvT1 hydrogenosomes

   8.7 ml PBS, 1 mM $MgCl_2$, 10 mM imidazole

E. rec. His- FOO only:

1 ml matrix

250 µl FOO in 400 mM imidazole

8.7 ml PBS, 1 mM $MgCl_2$

Columns were closed and incubated for 1 hour at RT on shaker. Then the matrix was let to settle and the columns were drained. The columns were rinsed twice with 4 ml of Wash buffer (section 4.1) and FOO protein was eluted 4x 0.5 ml of Elution buffer (section 4.1).

Eluted fractions were analyzed by SDS-PAGE and Western blot analysis.

5. Results

5.1 Purification of FOO

Recombinant FOO protein was produced in M15 *E. coli* cells (M 15 *E. coli* transformed with pQE 30 plasmid carrying FOO gene with 6xHis-tag on N-terminus) induced with IPTG. (Cloning into pQE plasmid and M15 *E. coli* cells transfection was done by I. Hrdý, Department of Parasitology, Charles University in Prague).

Recombinant His-FOO was purified from bacterial lysate by affinity chromatography on Ni-NTA column using stepwise gradient of 20 and 400 mM imidazole (Fig. 12). This purification is based on matrix affinity to polyhistidine tag, which is the component of recombinant His-FOO protein. Purity of isolated His-FOO was checked by SDS-PAGE (Fig. 13). Analysis of rec. His-FOO absorbtion spectrum revealed typical flavodiiron spectral structure (Fig. 14).

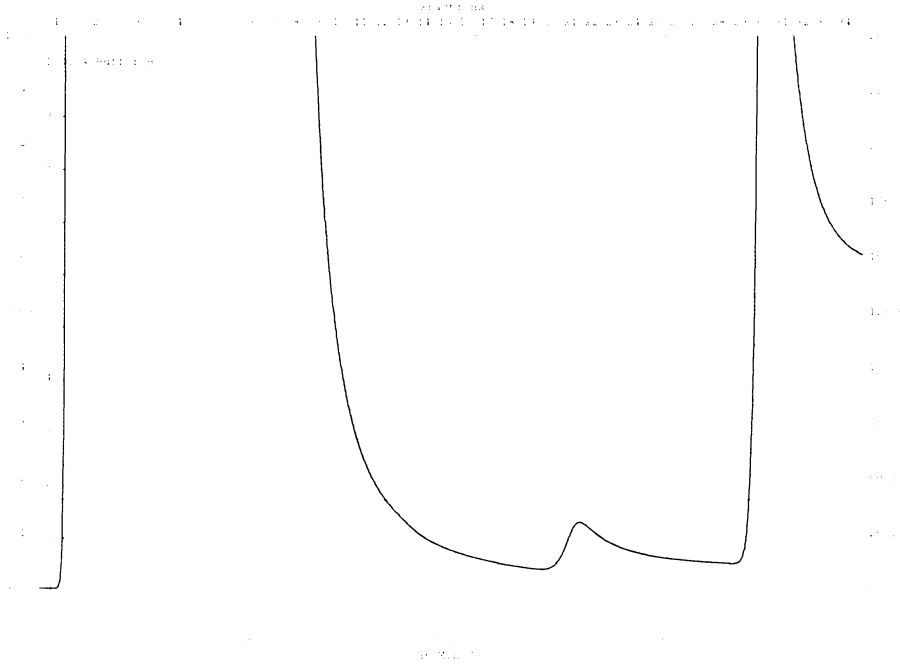


Figure 12. Ni-NTA purification of recombinant TvFOO by affinity chromatography on NiNTA column. 30, 31, fractions enriched in FOO; AU, absorbance units; blue line, absorbance of protein at 280 nm; red line, conductivity of elution buffer; black line, gradient of imidazole.

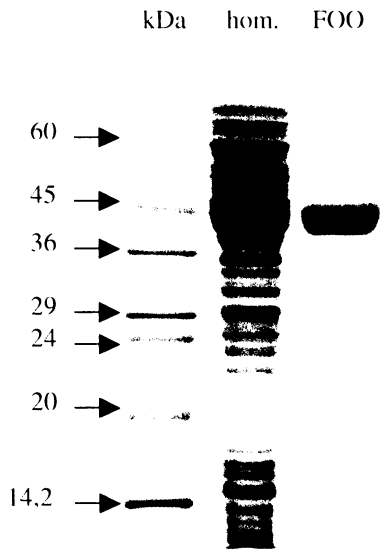


Figure 13. SDS-PAGE (12% gel). kDa, molecular weight standards; hom., bacterial lysate; FOO, purified TvFOO.

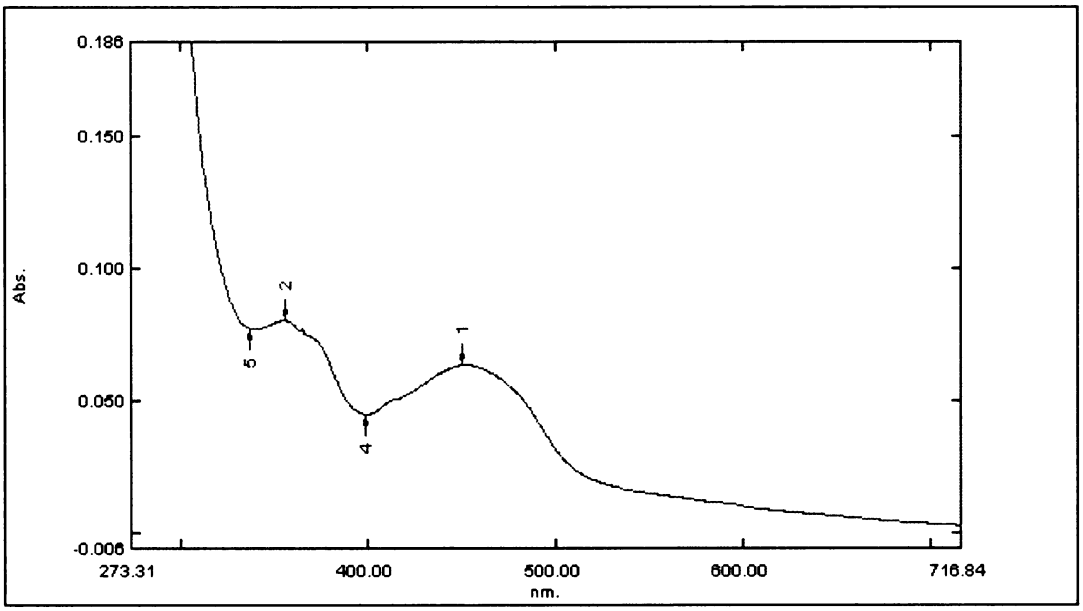


Figure 14. UV-VIS spectrum of rec. His-FOO protein. 1,2, characteristic bands of flavin cofactor.

5.2 Determination of native molecular weight of FOO

FOO polypeptide migrates on SDS-PAGE as a band of molecular mass of about 45 kDa (Fig. 13). Flavodiiron proteins present in nature are functional dimers. For determination of native molecular mass, I used hydrogenosomal lysate from transformed *T. vaginalis* overexpressing FOOWT and recombinant His-FOO isolated from *E. coli* by Ni-NTA affinity chromatography (Fig.12). Native molecular mass of rec. His-FOO determined by gel filtration chromatography (Superdex 75 column) (Fig.16) was approximately 92 kDa. Calibration curve of column is shown in Fig. 15. Position of FOOWT protein present in hydrogenosomal lysate in elution profile was determined by SDS-PAGE (Fig.17) and Western blot probed with specific polyclonal antiserum against FOO (Polyclonal antiserum against FOO was prepared in laboratory of Doc. Lukeš, Institute of parasitology Academy of Sciences of the CZ, České Budějovice). Again, the FOOWT eluted at approximately the same position as purified recombinant protein (peak in fraction 8 in elution profile, Fig. 18), indicating that FOO is dimer in vivo.

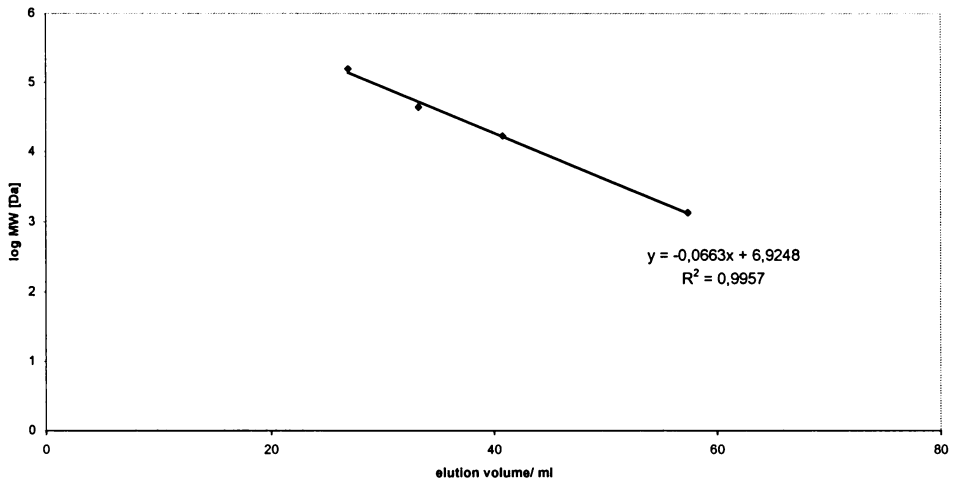


Figure 15. Calibration curve of Superdex 75 column with molecular weight standards (paragraph 4.5, Table 1). The equation was used for calculation of FOO native molecular mass.

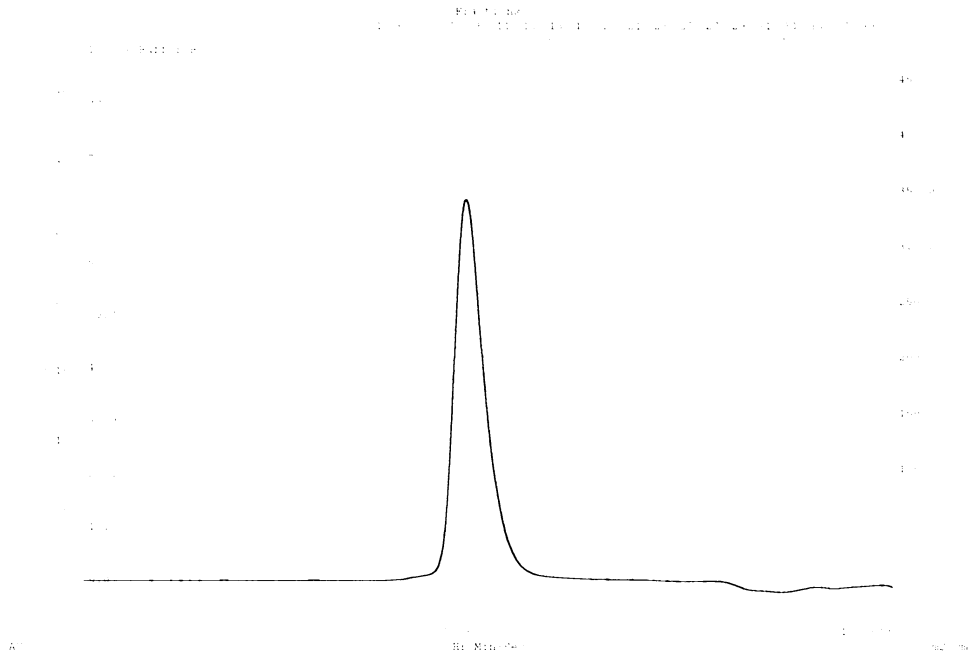


Figure 16. Elution profile of rec. His-FOO on gel filtration chromatography (Superdex 75 column). Blue line, absorbane of protein at 280 nm (AU, absorbane units); Red line, conductivity of elution buffer.

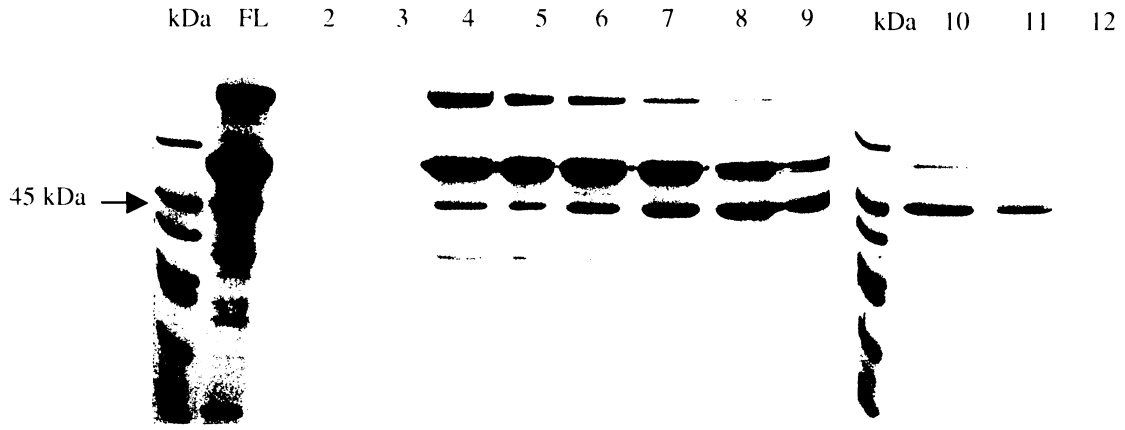


Figure 17. SDS-PAGE (12% gel) analysis of Superdex 75 fractions of hydrogenosomes from TvFOOWT. kDa, molecular weight standards; FL, sample loaded; 2-12, eluted fractions.

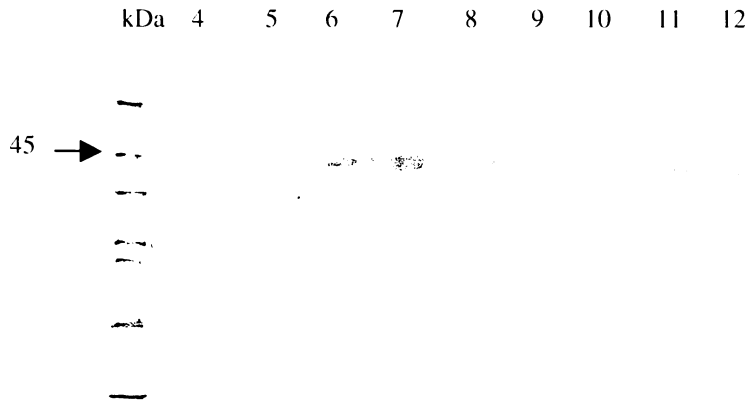


Figure 18. Western Blot analysis of fractions from Superdex 75 column, loaded with TvFOOWT solubilized hydrogenosomes (fractions 4-12). kDa, molecular weight standards; Anti FOO polyclonal antiserum was used for detection of FOO protein.

5.3 Determination of FOO cofactor

The type of flavin cofactor present in FOO was determined by TLC. FMN and FAD (0,6 mM) were used as standards. The spots were detected under the UV lamp (Fig. 8). The retention factor of cofactor from FOO protein (Rf FOO) was compared with retention factors of standards (Rf FMN and Rf FAD).

Rf FMN = 0,462

Rf FAD = 0,264

Rf FOO = 0,467

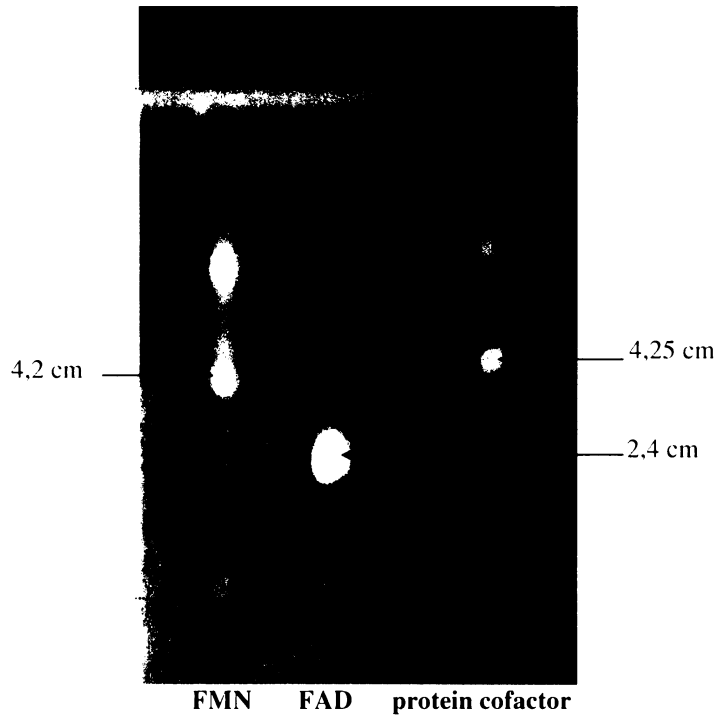


Figure 19. TLC: spots with migration distance: FMN, FMN standard; FAD, FAD standard; protein cofactor, cofactor released from FOO.

From the chromatogram and Rf value, it is apparent that FOO cofactor migrates at the rate comparable to the one of FMN. This suggests the presence of FMN cofactor in FOO protein, like in majority of flavodiiron proteins.

5.4 Preparation of MasterNEO transfection vector encoding for FOO

For the study of FOO function in hydrogenosomal metabolism, *FOO* gene was cloned into *T. vaginalis* transfection vector MasterNEO (MN). Positive transformants carrying plasmid were selected by treating with geneticin and the presence of *FOO* was verified by Western blot analysis in cell fractions obtained by differential centrifugation.

Three different transfection plasmids were constructed: Plasmid MNFOO WT codes for the native *FOO* with hydrogenosomal leader sequence at N-terminus. Plasmids MNFOO His and MNFOO HaHa contain *FOO* gene possessing, in addition to the hydrogenosomal leader sequence at N-terminus, *His* or *Haha* tag sequence at C-terminus, respectively. FOO gene was amplified by PCR using DNA from *T. vaginalis* gDNA library (XLOR cells, lambda ZAP vector; I. Hrdý, Department of Parasitology, Charles University in Prague) as a template (Fig. 20). PCR product was cloned into pGEM-T Easy vector and subsequently into relevant MN vector (Fig. 21). Protein expression in transfected trichomonads was proved by Western blot and immunodetection (Fig. 22, 23). Expression of FOOHis could not be detected probably in due to lowered expression of His-tagged proteins.

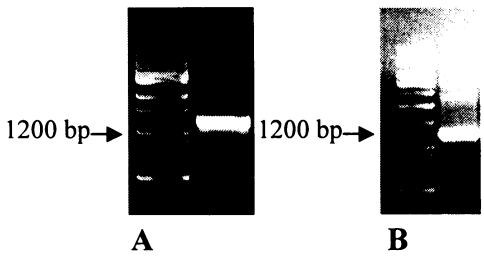


Figure 20. PCR amplification of FOO gene (4.8.1.1). A, *FOO* amplification with primers FOO *NdeI* F and FOO Stop *BamHI* R; B, *FOO* amplification with FOO *NdeI* F and FOO *Acc65I* R.

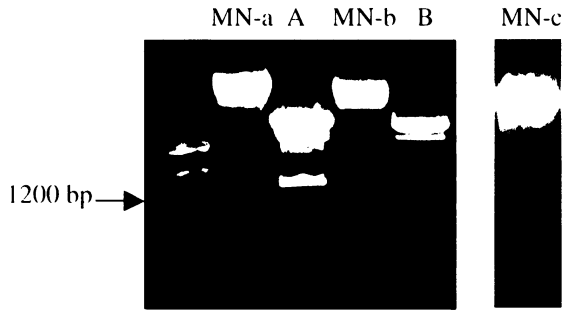


Figure 21. Linearization of MN and cleavage of *FOO* from pGEM-T easy vector by restriction endonucleases. MN-a, plasmid MN restricted with *NdeI* and *BamHI*; MN-b, pMN (+His) retricted with *NdeI* and *Acc65I*; MN-c, pMN (+Haha) restricted with *NdeI* and *Acc65I*; A, pGEM-*FOOWT* with cleaved *FOO* (*NdeI*;*BamHI*); B, pGEM-*FOOHis/HaHa* (*NdeI*, *Acc65I*).

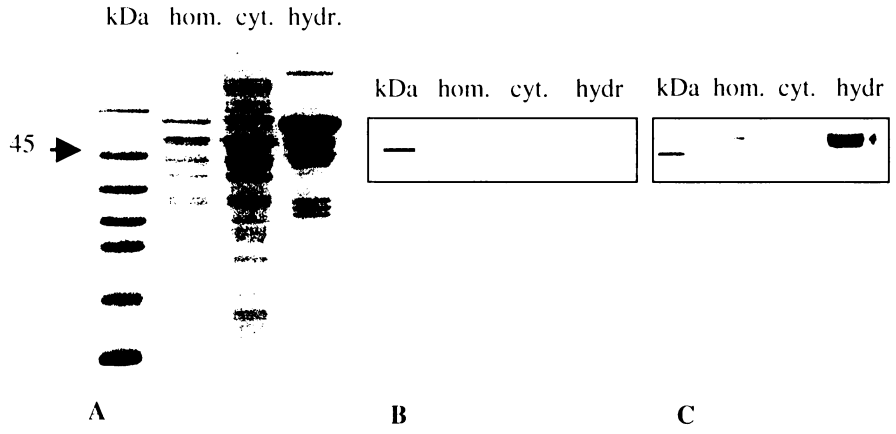


Figure 22. Subcellular localisation of FOO expressed in *T. vaginalis* line transformed with pMN(+HaHa) containing *FOO* insert (TvFOOHaHa). Panel A, SDS-PAGE (12% gel) of subcellular fractions. kDa, molecular weight standards, hom., homogenate, cyt., cytoplasm, hydr., hydrogenosomes; Panel B, Western blot with anti FOO polyclonal antiserum; Panel C, Western blot with antibody against HaHa tag.

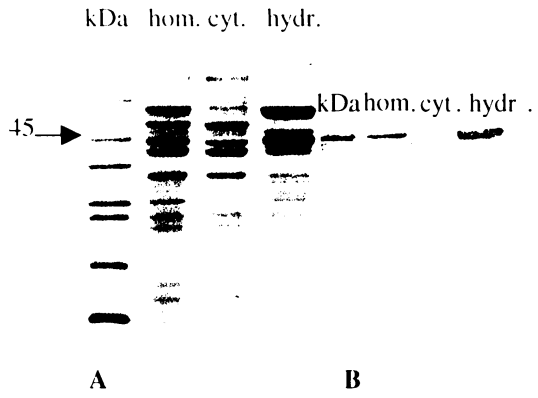


Figure 23. Subcellular localisation of FOO expressed in *T. vaginalis* line transformed with pMN containing *FOO* insert without tag sequence (TvFOOWT). Panel A, SDS-PAGE (12% gel) of subcellular fractions: kDa, molecular weight standards; hom., homogenate; cyt., cytoplasm; hydr., hydrogenosomes; Panel B, Western blot with polyclonal antiserum against FOO protein.

5.5 Preparation of TagVag 3 plasmid and TvFOOStrep cell line

For construction of plasmid TV 3 containing short sequence of *Strep*-tag, TagVag 2 plasmid with *HaHa* tag was used. *HaHa* tag sequence was cleaved off and replaced with *Strep*-tag (paragraph 4.9.1) (Fig. 24).

FOO gene was amplified by PCR and inserted into plasmid TV 3. Expression of FOO protein in the cells transformed by electroporation (TvFOOStrep) were checked by Western blot with anti FOO polyclonal antiserum and anti Strep antibody (Fig. 25).

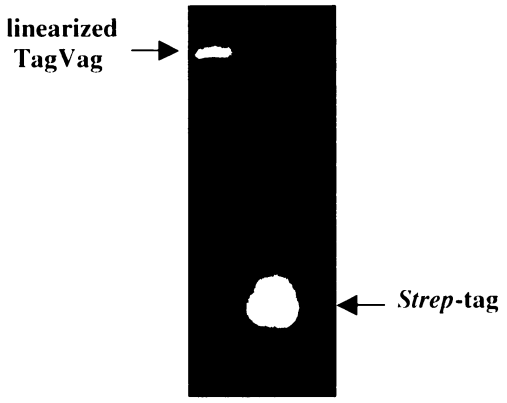


Figure 24. Preparation of TV3 vector. Linearized TagVag plasmid (2 µl) before ligation. *Strep*-tag insert (2µl) obtained by pairing of *strep* F and *strep* R bases.

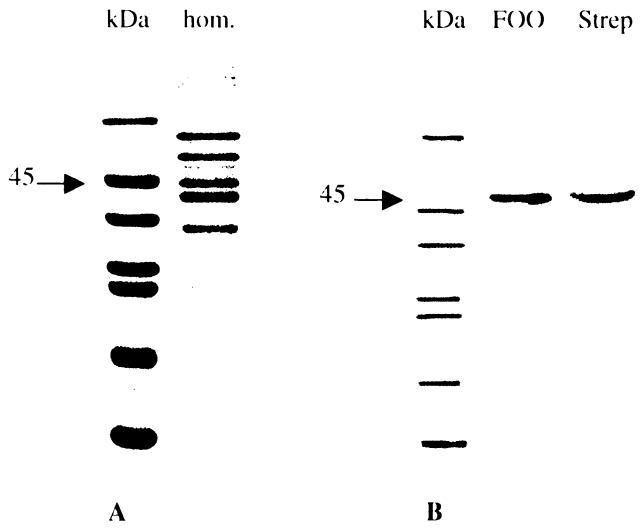


Figure 25. Detection of FOO expession in the homogenate (hom.) of *T. vaginalis* line TvFOOStrep. Panel A, SDS-PAGE (12% gel); Panel B, Western blot with anti FOO polyclonal antiserum (FOO) and anti Strep-tag (Strep) antibody; kDa, molecular weight standards.

5.6 Localisation of FOO protein in *T. vaginalis* cell by immunofluorescence

Trichomonas vaginalis transformed with plasmid MN containing *HaHa* tag (TvFOOHaHa) were used for preparation of mounts for immunodetection of FOO (paragraph 4.3.7.2). Antibody against HaHa tag and hydrogenosomal malic enzyme polyclonal antiserum were used as primary antibodies. Malic enzyme (secondary antibody with Alexa RED) is used as hydrogenosomal marker (Fig. 15B). FOO (secondary antibody with Alexa GREEN) (Fig. 15C) was localized in the same compartment of the cell as malic enzyme (Fig. 15D). Therefore, FOO is component of hydrogenosomes, consistent with the presence of hydrogenosomal leader sequence at the N-terminus of nascent FOO protein.

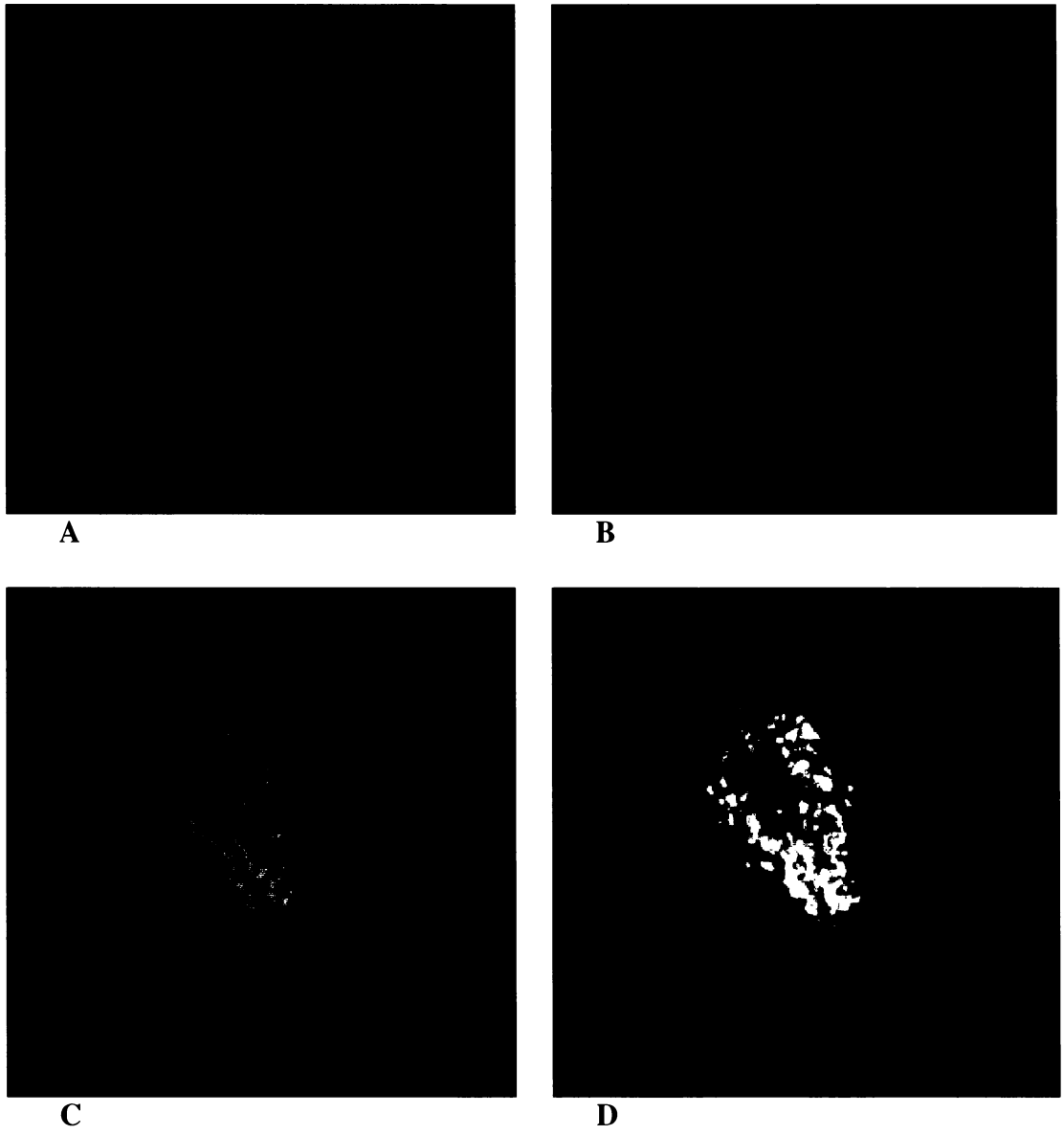


Figure 26. Immunodetection of FOO in *T. vaginalis* cell: A, DAPI staining of nucleus; B, detection of malic enzyme, localization in hydrogenosomes; C, FOO labelling; D, displacement of colour channels suggesting presence of FOO in hydrogenosomes.

5.7 Purification of Tv ferredoxin

Recombinant TvFdx was isolated for the purpose of respiration experiments with purified His-FOO in isolated system. The working hypothesis was that ferredoxin could serve as electron donor for FOO (ferredoxin:oxygen oxidoreductase).

TvFdx, produced in BL21 *E. coli* cells (paragraph 4.3.11) was purified by two steps of liqiud chromatography. First purification step was done by anion-exchange chromatography on DEAE-Sephacel column eluted with two-step linear gradient of NaCl (Fig. 27). Concentrated fractions containing TvFdx were loaded onto Superose 12 and purified by isocratic flow (Fig. 28). Fractions containing TvFdx were of red-brown colour.

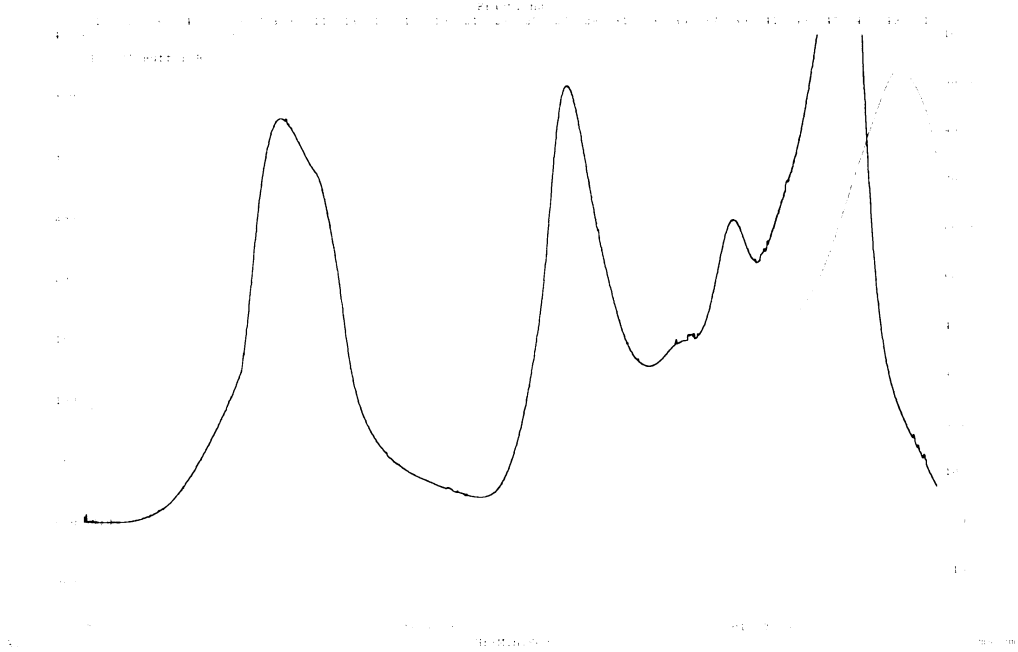


Figure 27. DEAE Sephacel chromatography: First step of recombinant TvFdx purification from bacterial lysate by anion-exchange chromatography on DEAE Sephacel column. Fractions 36-40 are enriched in ferredoxin; Blue line, absorbance of protein at 280 nm; AU, absobance units; Red line, conductivity of elution buffer; Black line, gradient of NaCl.

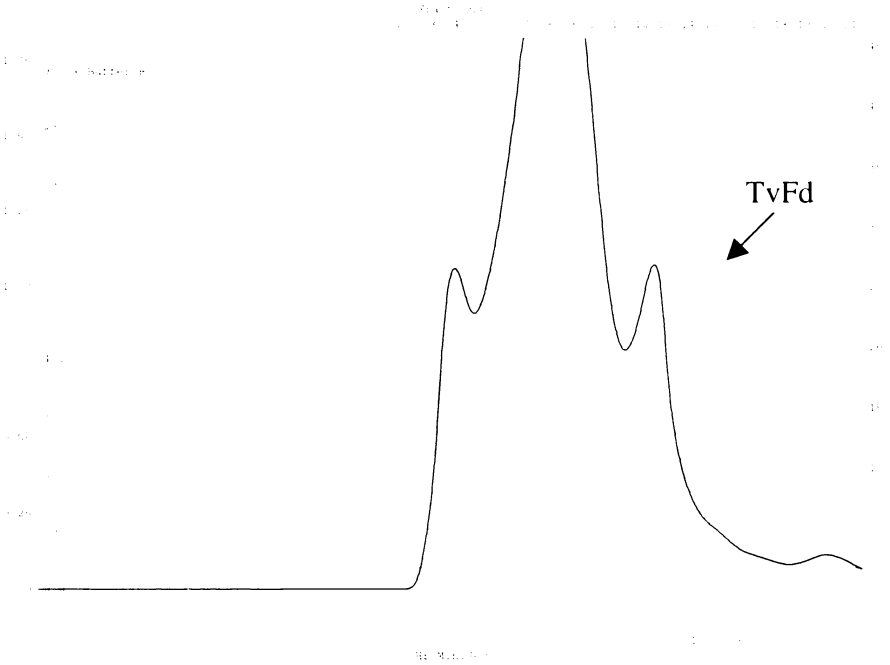


Figure 28. Superose 12: Second step of recombinant TvFdx purification by liqiud chromatography on Superose 12 column. Blue line, absorbance of protein at 280 nm; AU, absobance units; Red line, conductivity of elution buffer.

5.8 The search for possible FOO – interacting hydrogenosomal proteins

To elucidate the role of FOO in the hydrogenosome, I have attempted to identify the potential FOO – interacting proteins by means of immobilization of rec. His-FOO on Ni-NTA agarose (QiaGen), incubation with hydrogenosomal extract and specific elution of potential protein complex with 400 mM imidazole.

After incubation with TvT1 hydrogenosomal extract (TvT1 hydrogenosomes were treated with detergents by experimental conditiones described in paragraph 4.10), FOO was isolated using Poly-Prep chromatography columns (BioRad) with Ni-NTA agarose and the eluted fractions were investigated for the presence of other proteins with potential role as electrontransport partners of FOO participating in hydrogenosomal metabolism. Elution was done with 4x 0.5 ml 400 mM imidazole elution buffer and two first elution volumes were analyzed by SDS-PAGE (Fig. 29) and Western blot (Fig. 30).

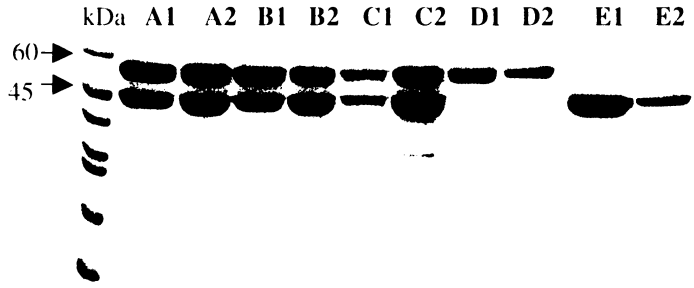


Figure 29. SDS-PAGE (12% gel) of rec. His-FOO immobilized on Ni-NTA column, incubated with hydrogenosomal extract and eluted with 400 mM imidazole. kDa, molecular weight standards; A, hydrogenosomes treated with 2% octylglucoside; B, hydrogenosomes treated with 1% TX 100; C, hydrogenosomes treated by freezing / thawing; D, hydrogenosomes only; E, FOO only; 1, first elution; 2, second elution.

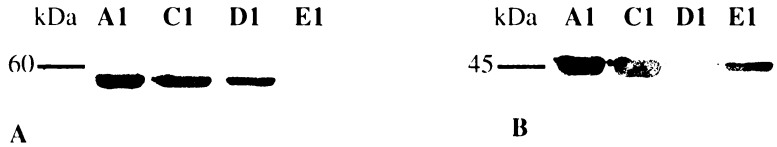


Figure 30. Western blot analysis of proteins eluted with 400 mM imidazole from Ni-NTA column with bound rec. His-FOO and incubated with hydrogenosomal extract: Panel A, anti malic enzyme polyclonal antiserum; Panel B, anti FOO polyclonal antiserum, A1, hydrogenosomes treated with 2% octylglucoside; C1, hydrogenosomes treated by freezing / thawing; D1, hydrogenosomes only; E1, FOO only; kDa, molecular weight standards.

There are two thick and some thin bands on SDS-PAGE (Fig. 29). Western blot analysis and immunodetection of hydrogenosomal enzymes (STK, PFO, ME, Fdx, FOO and Complex I) with specific antibodies detected only malic enzyme (ME) as polypeptide migrating on SDS-PAGE as a band of molecular mass of about 60 kDa and FOO protein migrating as a band of molecular mass of about 45 kDa (Fig. 30). The other bands are probably residues from bacterial lysate, which rec. His-FOO was purified from. This hypothesis is confirmed by the presence of thin bands in the lines containing rec. His-FOO only (Fig. 29, line E1, E2). As apparent from lines D and D2 (Fig. 29), retention of ME on Ni-NTA matrix is not related to the presence of FOO. Therefore, it appears that no hydrogenosomal FOO-interacting proteins could be identiefied using this experimental approach.

5.9 Expression of FOO in *T. vaginalis* resistant to metronidazole

Metronidazole-resistant trichomonads have altered expression and activities of hydrogenosomal enzymes participating in the activation of the drug. These trichomonads are also very sensitive to oxidative stress [5]. Therefore, it was of interest to see whether the level of expression of FOO is altered in metronidazole-resistant cells. The level of FOO expression was determined in metronidazole sensitive strain TV 10-02 and its resistant derivative TV 10-02 MR100 using Western blot and immunodetection with specific anti FOO antibody (Fig. 31).

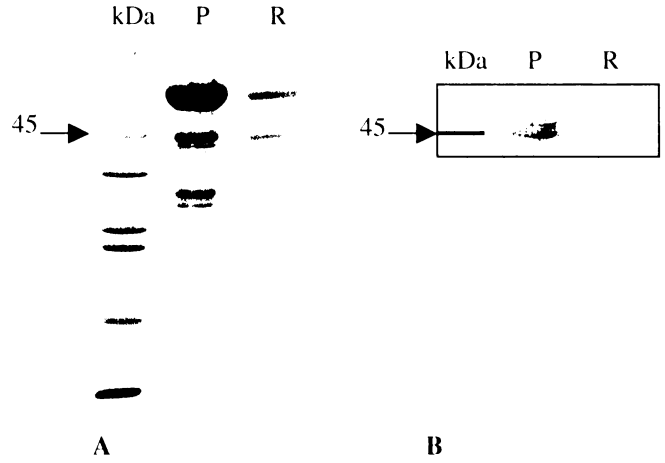


Figure 31. Panel A, SDS-PAGE (12% gel); Panel B, Western blot analysis (anti FOO polyclonal antiserum) comparing FOO expression in metronidazole-susceptible (P) and metronidazole-resistant (R) trichomonads; kDa, molecular weight standards.

Although it is difficult to balance the protein load on SDS-PAGE, as the resistant trichomonads lack dominant hydrogenosomal proteins such as ME and PFO, it is apparent that the expression of FOO in resistant trichomonads is markedly reduced (Fig. 31).

5.10 Determination of hydrogenosomal respiration

Hydrogenosomal respiration was measured with the organelles isolates from TvT1 and TvFOOWT. Determination of activity was repeated four times with independent hydrogenosomal isolates. The respiration rate was measured as the decline of oxygen concentration in the detection system using Clark-type oxygen electrode. NADH-dependent and pyruvate-dependent respiration of hydrogenosomes was monitored. Specific activities (respiration rates) of NADH- (Figs. 33, 36) and

pyruvate-dependent (Figs. 34, 36) pathways of TvT1 and TvFOOWT cell lines were compared.

Hydrogenosomes from TvT1 line transfected with plasmid containing gene for subunit 6 (TvSUB6), subunit of putative membrane domain of Complex I, were used as a control for transformed trichomonads in the measurements (cells gently provided by Lucie Bardoňová, Department of Parasitology, Charles University in Prague). Gene for SUB6 was cloned in pMN with HaHa tag. This protein is apparently strongly hydrofobic and its expression in trichomonad cells could not be detected. Level of FOO expression in trichomonad lines was checked by SDS-PAGE and Western blot with anti FOO polyclonal antiserum (Figs. 32, 35).

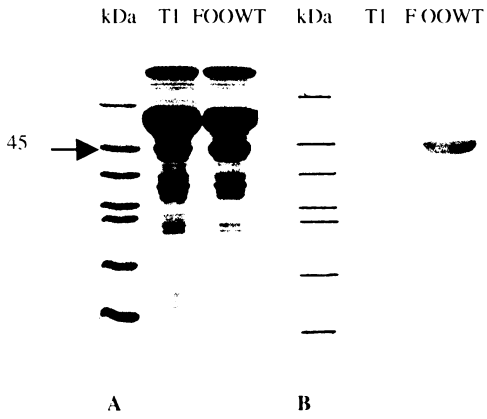


Figure 32. Comparison of FOO expression in hydrogenosomes TvT1 (T1) and TvFOOWT (FOO WT) line by SDS-PAGE (Panel A) (12% gel) and Western blot with anti FOO polyclonal antiserum (Panel B). kDa, molecular weight standards.

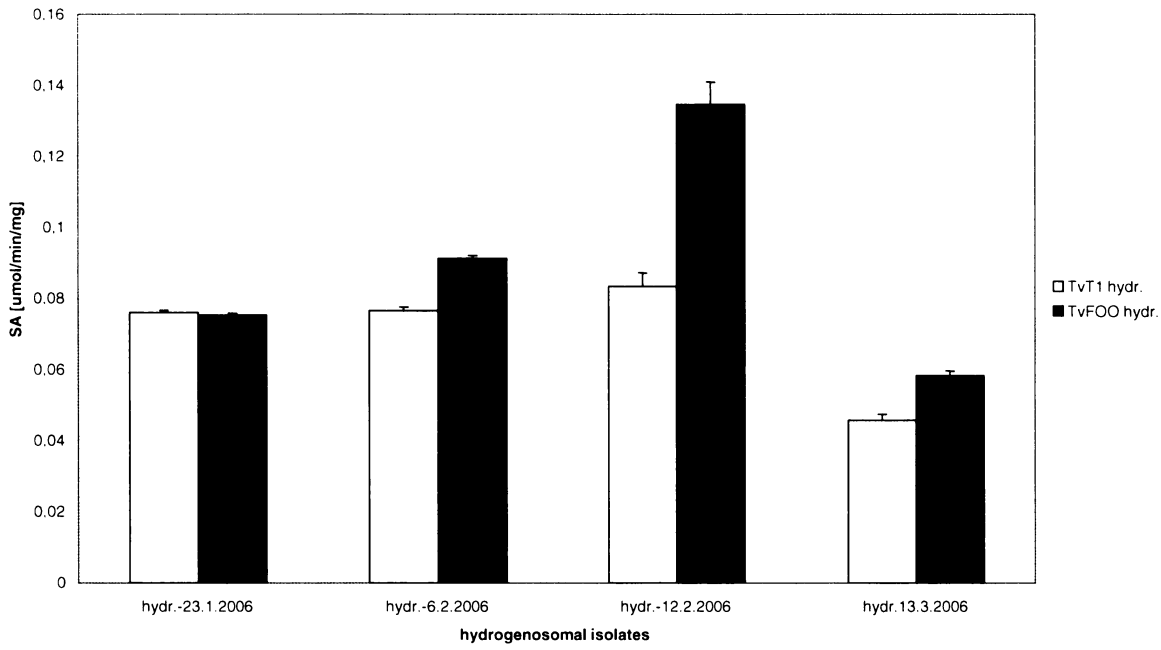


Figure 33. Comparison of NADH-dependent hydrogenosomal respiration in four independent organelle isolations from TvT1 and TvFOOWT cell line.

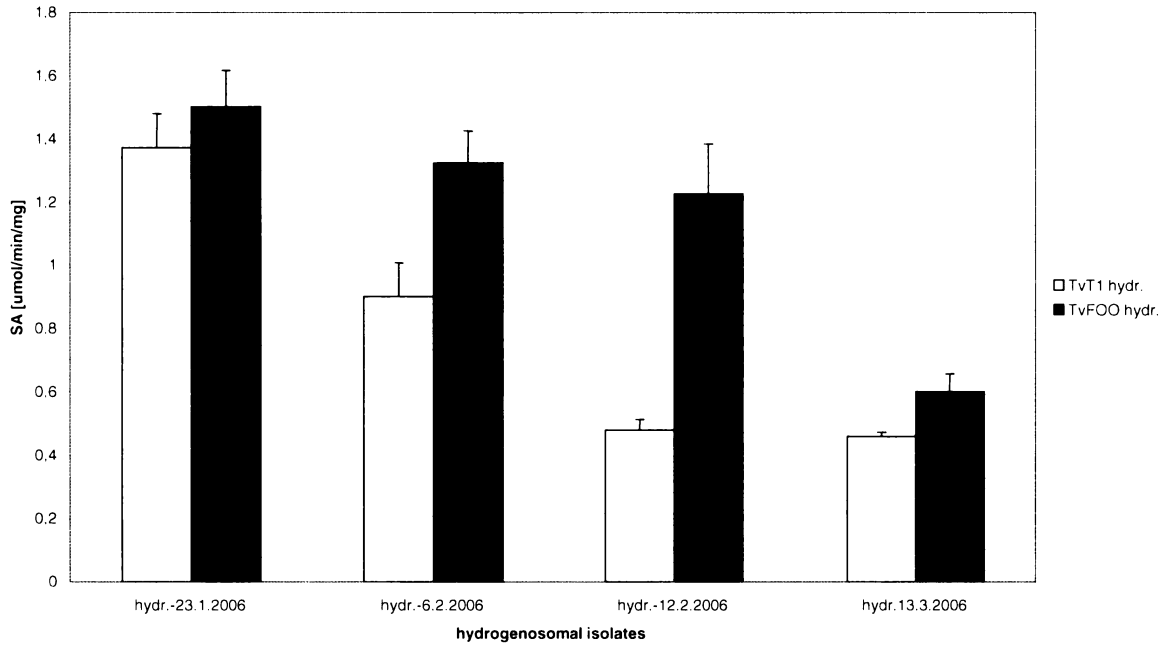


Figure 34. Comparison of pyruvate-dependent hydrogenosomal respiration in four independent organelle isolations from TvT1 and TvFOOWT cell line.

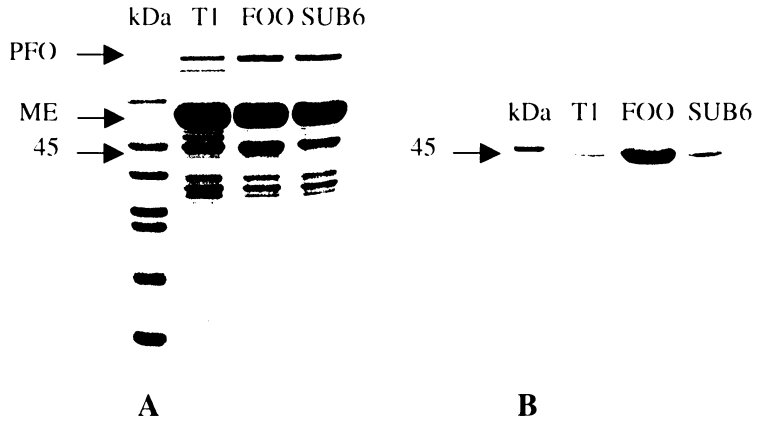


Figure 35. Panel A, SDS-PAGE of hydrogenosomes from TvT1 (T1), TvFOO (FOO) and TvSUB6 (SUB6) isolated by differential centrifugation on 13.03.2006; kDa, molecular weight standards; ME, malic enzyme; PFO, pyruvate:ferredoxin oxidoreductase; Panel B, comparison of FOO expression in hydrogenosomes analyzed by Western blot with polyclonal antiserum against FOO protein.

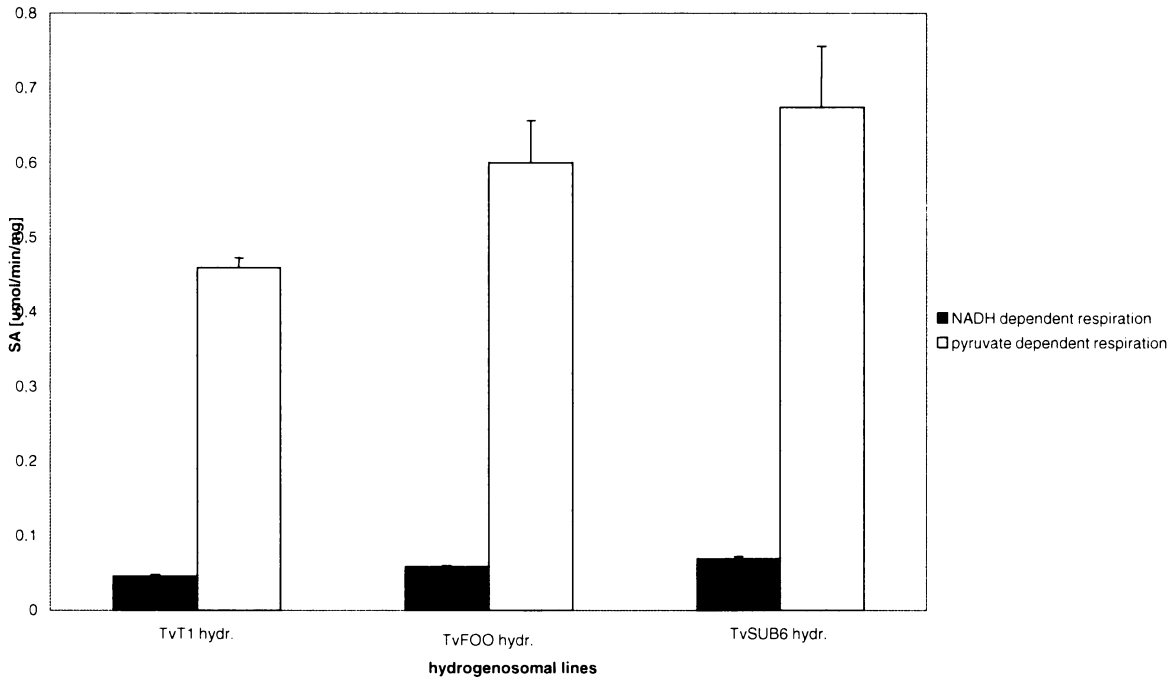


Figure 36. Comparison of hydrogenosomal respiration using hydrogenosomes isolated from TvT1, TvFOOWT and TvSUB6 cell line on 13.03.2006. Both NADH-dependent (orange) and pyruvate dependent (green) respiration rates are compared.

Hydrogenosomes of TvFOOWT cells overexpressing FOO display increased consumption of oxygen in both NADH- and pyruvate-dependent respiration pathways. However, the increased respiration rate is also observed in the hydrogenosomes isolated

from TvSUB6 line. These results indicate that FOO overexpression in trichomonad cell doesn't confer provable respiration phenotype with the electron donors used.

5.11 Determination of respiration with rec. His-FOO

Respiration activity of purified rec. His.-FOO was measured in isolated system using Clark-type oxygen electrode. Purified Complex I (L. Bartoňová, Department of Parasitology, Charles University in Prague) was used for NADH-dependent reduction of ferredoxin. Reduced ferredoxin was supposed to interact with FOO and serve as electron donor in the process of hydrogenosomal respiration. However, due to its instability in salt-free solutions, rec. His-FOO had to be used a- purified in 400 mM imidazole, which caused inhibition of oxygen consumption even by autooxidation of ferredoxin upon FOO addition into the electrode chamber (Fig.37).

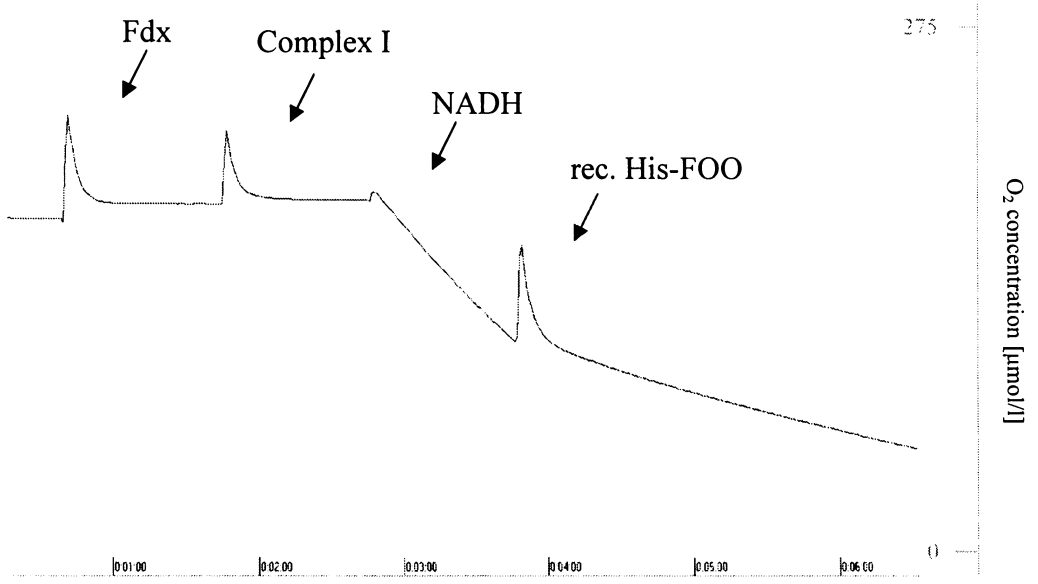


Figure 37. Respiration with rec. His-FOO protein. Fdx, ferredoxin; Complex I, homologue of mitochondrial Complex I; rec. His-FOO, purified recombinant His-FOO in 400 mM imidazole.

5.12 Determination of enzymatic activities in isolated hydrogenosomes

In order to elucidate the possible phenotype related to overexpression of FOOWT in trichomonad hydrogenosomes, I have determined the activities of several major hydrogenosomal enzymes participating in redox reactions: hydrogenase (Fig.39), malic

enzyme (ME) (Fig. 38), pyruvate:ferredoxin oxidoreductase (PFO) (Fig. 40) and Complex I (Fig. 41).

Comparison namely of activities of PFO and Complex I with respiration rates determined for hydrogenosomes supplied with pyruvate or NADH could reveal the involvement of overexpressed protein in electrontransporting pathways.

Hydrogenosomes from the line TvFOOWT were used. These cells produce FOO protein containing hydrogenosomal leader without tag sequence (wild-type protein) at several-times higher level than TvT1 (Fig. 35).Organelles from 1 liter culture were isolated and divided into aliquots kept at –70°C until used. The activity of (ME), (PFO), hydrogenase and Complex I was investigated. The activities were compared with those of TvT1 hydrogenosomes. Enzymatic activities from four independent organelle isolations were compared.

Activities of the same enzymes of TvSUB6 hydrogenosomes were determined as well.

Enzymatic activity of ME determined with TvFOOWT hydrogenosomes was reduced in comparison with activity of TvT1 organelles. In contrast, enhanced activities of PFO (Fig. 40) and Complex I (Fig. 41) in FOO-overexpressing cell line were detected. Activity of hydrogenase was comparable for both cell lines (Fig.39). The same trend of enzymatic activities has been detected in TvSUB6 (Fig. 42). The observed activities of at least ME and PFO correlated with the levels of the respective hydrogenosomal proteins as apparent on SDS-PAGE (Fig. 35).

These results indicate that changed respiration rates and enzymatic activities of TvFOOWT may be caused by selection of slightly different phenotypes following the transfection of trichomonads and probably do not relate to FOO overexpression.

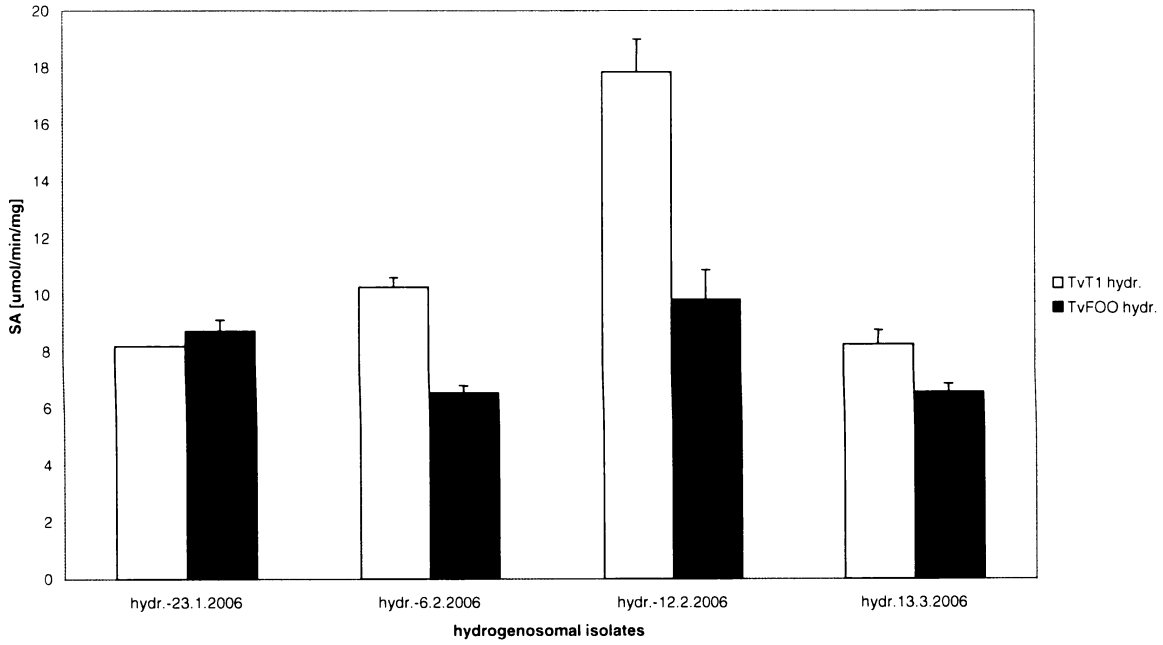


Figure 38. Determination of ME activity in hydrogenosomal isolates from four independent organelle isolations. Comparison of activity from TvT1 hydrogenosomes (TvT1 hydr.) with TvFOOWT hydrogenosomes (TvFOO hydr.).

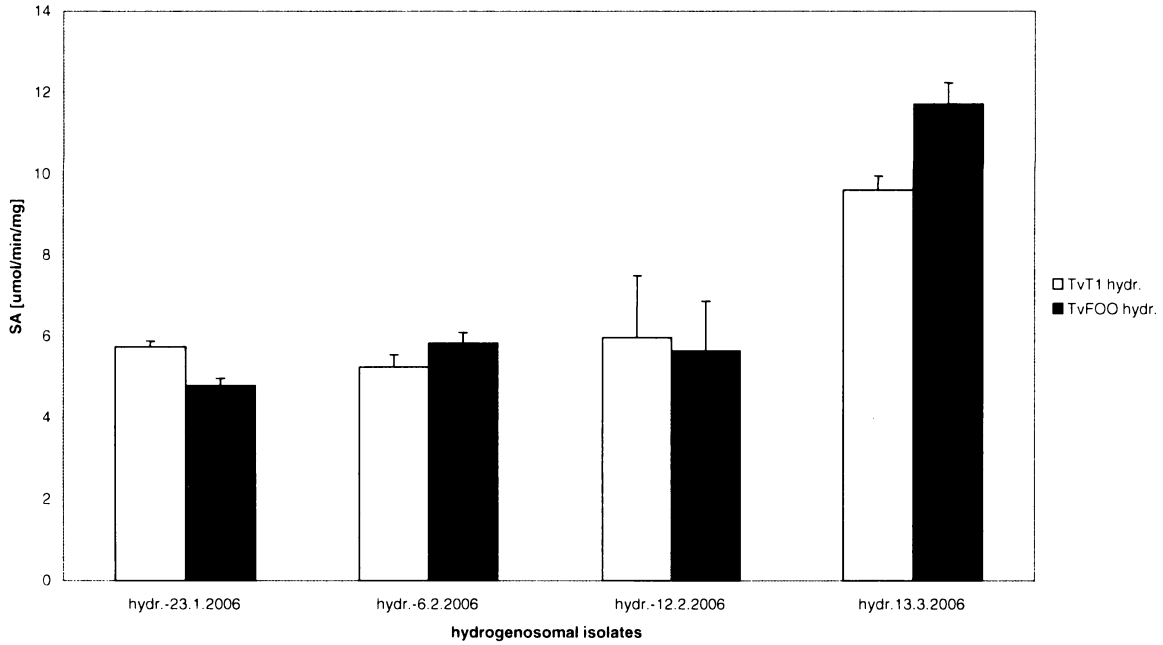


Figure 39. Determination of hydrogenase activity in hydrogenosomal isolates from four independent organelle isolations. Comparison of activity from TvT1 hydrogenosomes (TvT1 hydr.) with TvFOOWT hydrogenosomes (TvFOO hydr.).

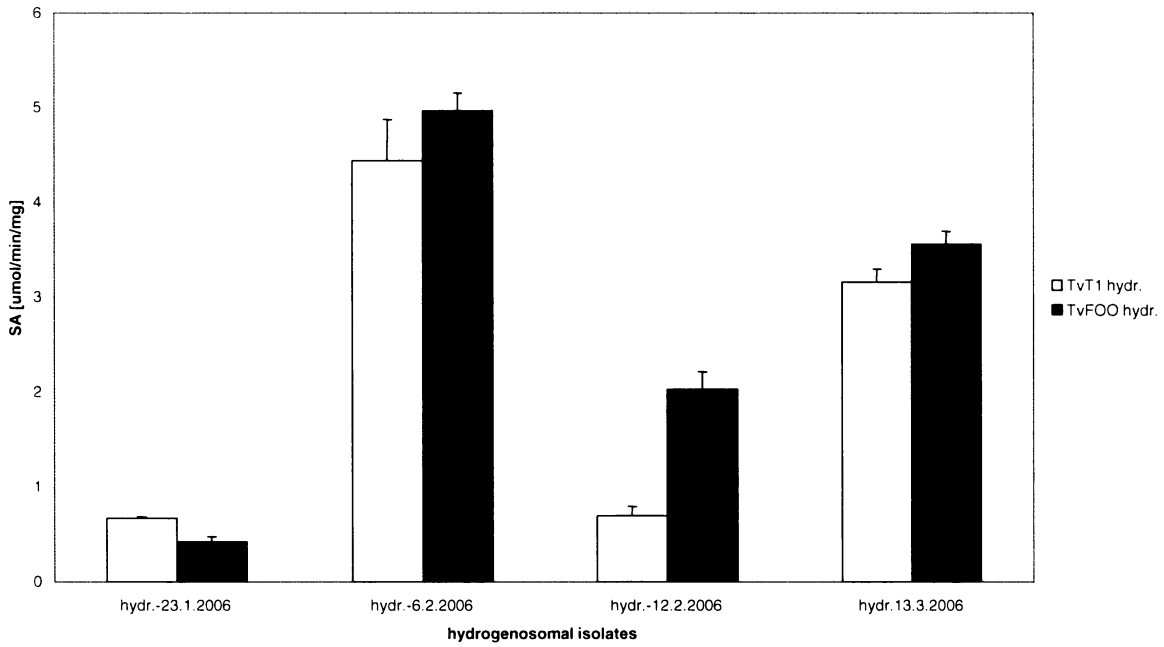


Figure 40. Determination of PFO activity in hydrogenosomal isolates from four independent organelle isolations. Comparison of activity from TvT1 hydrogenosomes (TvT1 hydr.) with TvFOOWT hydrogenosomes (TvFOO hydr.).

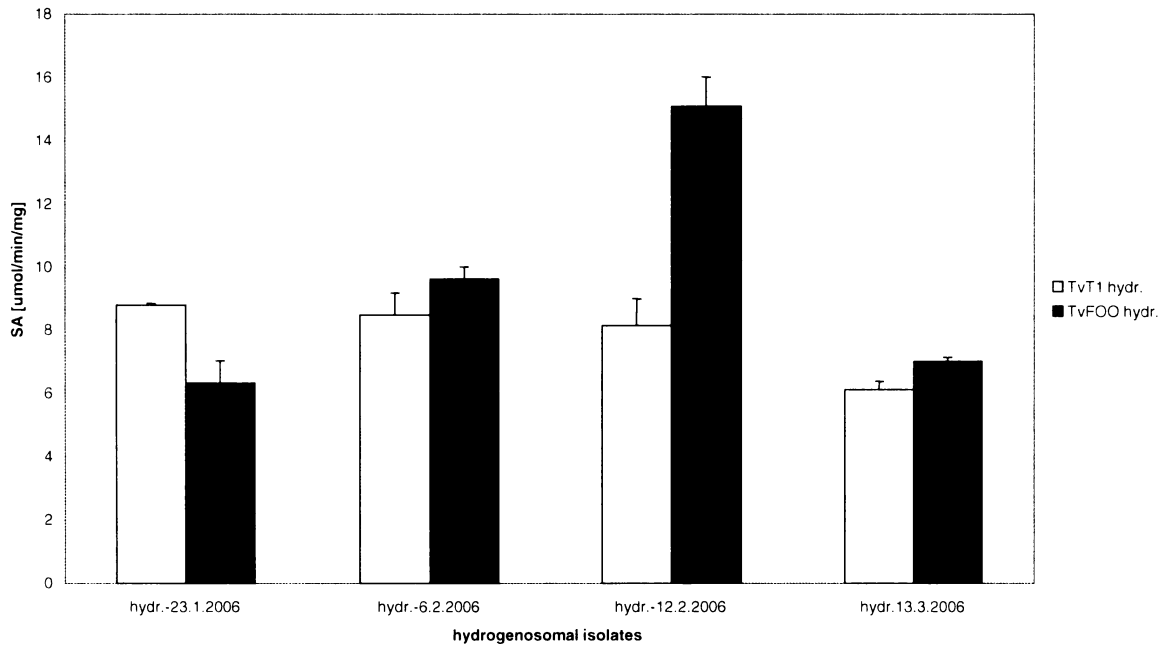


Figure 41. Determination of Complex I activity in hydrogenosomal isolates from four independent organelle isolations. Comparison of activity from TvT1 hydrogenosomes (TvT1 hydr.) with TvFOOWT hydrogenosomes (TvFOO hydr.).

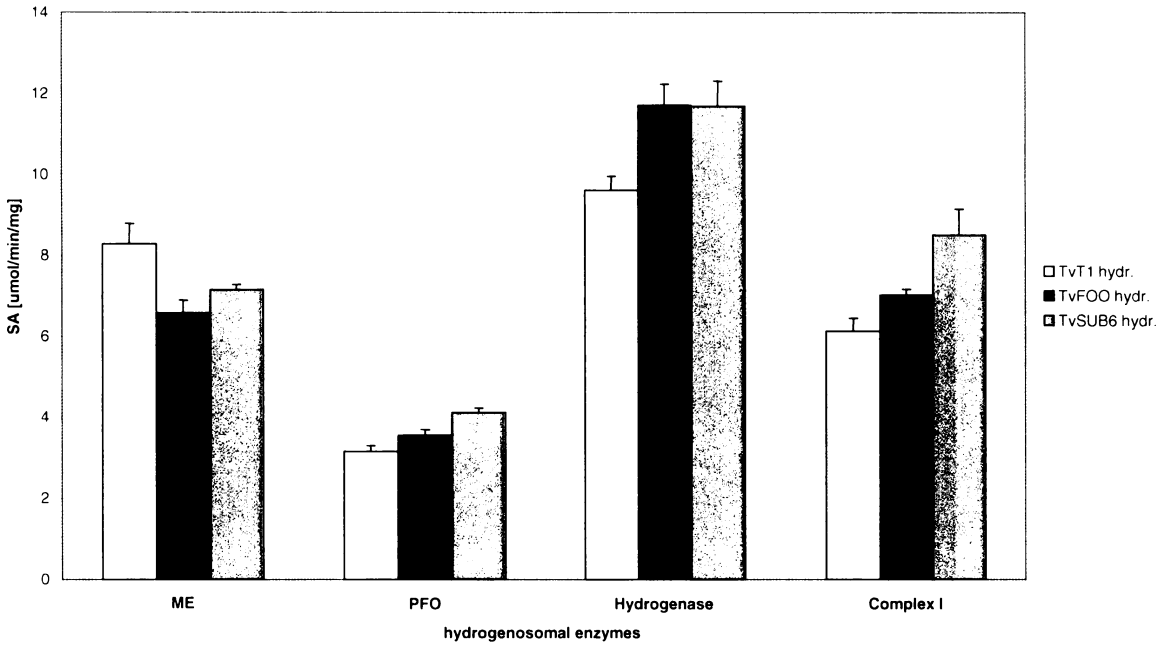


Figure 42. Comparison of enzymatic activities from TvT1 hydrogenosomes (TvT1 hydr.) with activities from TvFOOWT (TvFOO hydr.) and TvSUB6 (TvSUB6 hydr.) hydrogenosomes.

Comparison of NADH-dependent respiration (Fig. 31) and Complex I (Fig. 32) enzymatic activity correlated well in the three cell lines. Increased NADH-dependent respiration in transformed cells correlates with increased activity of Complex I. I suppose that increased NADH-dependent respiration is probably caused by increased expression of Complex I, which is one of the sources of reduced ferredoxin.

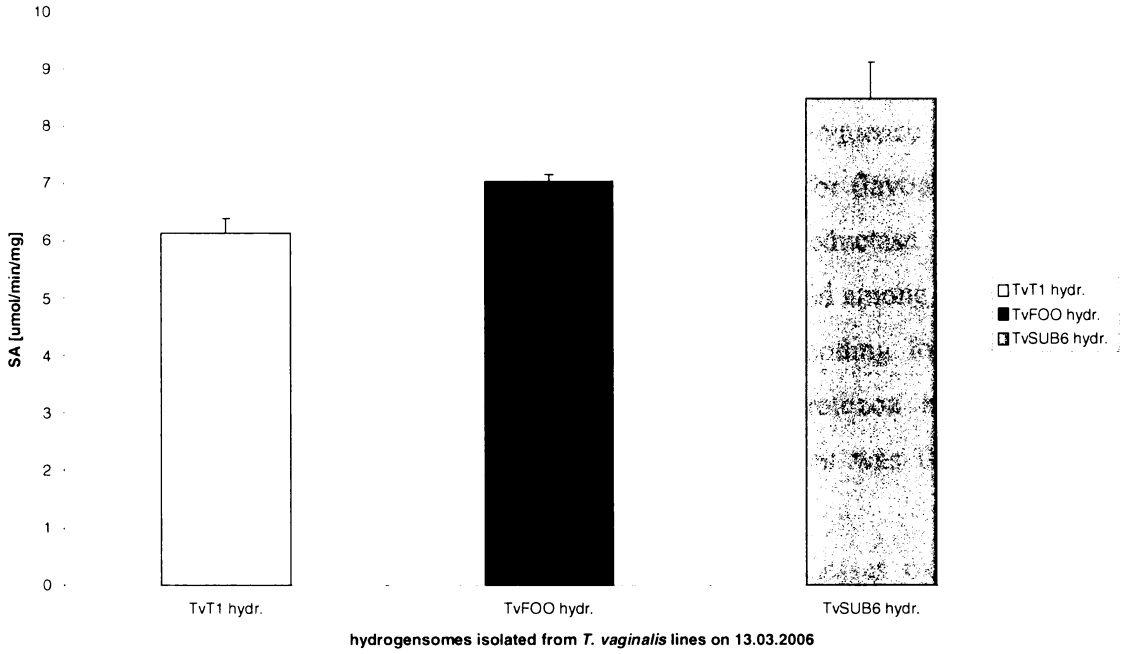


Figure 43. Comparison of Complex I activity determined with hydrogenosomal isolates from TvT1 (TvT1 hydr.), TvFOOWT (TvFOO hydr.) and TvSUB6 (TvSUB6 hydr.) cell lines.

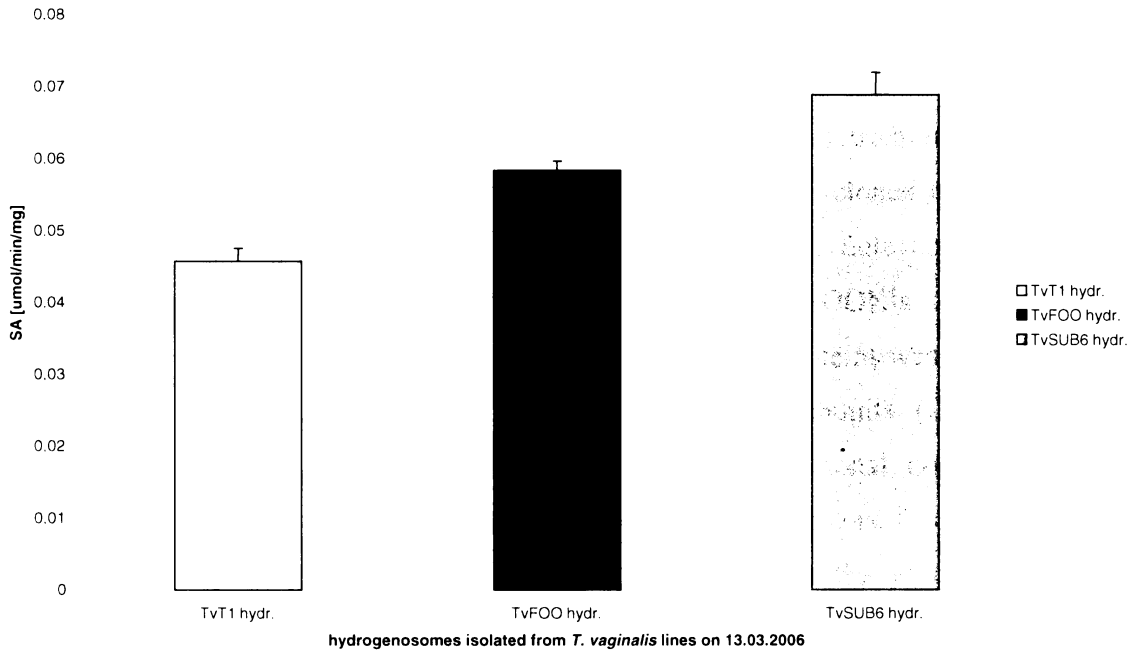


Figure 44. Comparison of NADH-dependent respiration determined with hydrogenosomal isolates from TvT1 (TvT1 hydr.), TvFOOWT (TvFOO hydr.) and TvSUB6 (TvSUB6 hydr.) cell lines.

6. Discussion

Ferredoxin:oxygen oxidoreductase (FOO) is the recently discovered flavoprotein in the genome of microaerophilic protozoon *Trichomonas vaginalis* (http://www.tigr.org/tdb/e2k1/tvg/). Analysis of FOO protein sequence revealed the presence of β-lactamase-like and flavodoxin-like domains typical for flavodiiron proteins, and thus homology of FOO with rubredoxin:oxygen oxidoreductase (ROO) from *Desulfovibrio gigas* [89]. This superfamily of enzymes is widespread among anaerobic and facultative anaerobic prokaryotes, and the presence of genes encoding for homologous proteins in amitochondriate parasitic and some free-living protozoa has also been confirmed. [88,89] The first function assigned to these enzymes was that of oxygen reduction to water [90,91].

T. vaginalis inhabits oxygen-poor environment and possesses oxygen-sensitive enzymes such as pyruvate:ferredoxin oxidoreductase and hydrogenase [5]. Considering the known function of flavodiiron proteins, FOO could perform a role of an oxygen-detoxification enzyme by scavenging oxygen and thus relieve or prevent oxidative stress.

In order to study FOO role in hydrogenosomal metabolism, *T. vaginalis* lines overexpressing FOO were prepared. TvFOOWT line overexpressing FOO protein without tag (wild type) was used in the enzymatic measurements and respiration experiments with hydrogenosomes. TvFOOHaHa cells, overexpressing protein with HaHa-tag, were used for immunolocalization of FOO in trichomonad cell. TvFOOHis cell line was constructed in order to isolate overexpressed FOO with C-terminal His-tag from trichomonad cell by affinity chromatography. The FOO gene (with or without tag) was cloned in MasterNEO expression vector and used for transformation of trichomonads. Selection of positive transformants was successful, but FOOHis expression in TvFOOHis cells was not confirmed. Very low expression of His-tagged hydrogenosomal proteins was also observed in expression of homologues of mitochondrial Complex I subunits (L. Bardoňová, Department of Parasitology, Charles University in Prague, personal communication). TvFOOHis cells grew very slowly compared to other transfected strains. I assume that His-tagged construct could be harmful to the *T. vaginalis* cell so the parasites limit its expression. Because expression of FOO-His and its isolation from *T. vaginalis* cell failed, recombinant His-FOO protein produced in bacteria was used in protein characterization experiments.

Recombinant *T. vaginalis* His-FOO protein was isolated from M15 *E. coli* cells transformed with pQE 30 plasmid containing FOO gene with N-terminal 6xHis-tag (I. Hrdý, Department of Parasitology, Charles University in Prague). Protein was eluted from Ni-NTA column with 400 mM imidazole. The fact, that after elution the protein was not stable in solution and precipitated soon after purification complicated experimental measurements. Threfore, it was necessary to perform all experiments directly after purification. Another problem was that protein purified using 400 mM imidazole precipitated right after dilution, or upon transfer to another buffer. The necessity to keep the rec. His-FOO in 400 mM imidazole hampered respiratory experiments, as the 400 mM imidazole itself inhibited the respiration of the system consisting of NADH, Complex I and ferredoxin.

SDS-PAGE and immunoblot analysis determined FOO as a polypeptide with molecular mass of about 45 kDa (Fig.13, page 56). Gel filtration chromatography determined that FOO is a protein with native molecular mass of about 92 kDa (paragraph 5.2). These findings suggest that the native protein exists as a dimer. This is in agreement with recent publications demonstrating that majority of flavodiiron proteins are functional dimers with head-to-tail quarternary structure, bringing the FMN from one subunit in close proximity to the diiron site of other subunit, facilitating electron transfer. Monomer molecular mass of Class A flavodiiron proteins (having only the core domains) is approximately 45 kDa. [93]

The flavodoxin-like module of flavodiiron proteins harbours flavin moiety, always a FMN in so far studied proteins [89]. The cofactor present in FOO was determined by TLC method. Retention factor of FOO cofactor corresponded to that of FMN standard. There were 2 spots migrating in the lines of FMN standard and FOO cofactor (Fig.19, page 59). Retention factors of these spots were identical for both samples. The two-spot pattern of FMN could be caused by cofactor decomposition by mobile phase during separation process.

FOO protein possesses hydrogenosomal leader sequence. This short targeting sequence is necessary for protein translocation to hydrogenosomes [39]. FOO localization was verified by Western blot analysis of *T. vaginalis* subcellular fractions (Fig.22, page 61) and by immunofluorescence experiments using TvFOOHaHa line that expressed HaHa-tagged protein (Fig.26; page 64). The hydrogenosomes were visualised using polyclonal serum against malic enzyme (ME) – the hydrogenosomal marker (Fig.26B, page 64).

Merge of colour channels corresponding to malic enzyme and FOO protein confirmed hydrogenosomal localisation of FOO.

Regarding the characterization of FOO and its putative function in *T. vaginalis* metabolism, I tried to search for possible hydrogenosomal proteins interacting with FOO. These proteins may serve as electron donors for FOO, like rubredoxin for ROO in *Desulfovibrio gigas* [90,94] or high molecular weight rubredoxin (Hrb) for flavodiiron protein in *Moorella thermoacetica* [96]. Since rubredoxin is absent in *T. vaginalis* genome, and ferredoxin appears to be a dominant electrontransporting protein in trichomonad hydrogenosomes (7 distinct ferredoxin genes have been identified in *T. vaginalis* genome – http://www.tigr.org/tdb/e2k1/tvg/, I have hypothesized that ferredoxin might be an electron donor for FOO. By incubation of TvT1 hydrogenosomal lysate with purified rec. His-FOO and subsequent purification of rec. His-FOO by affinity chromatography, I have tried to identify the possible FOO-interacting proteins. SDS-PAGE identified two major bands of about 45 kDa and 60 kDa and some faint ones with molecular mass in the range of 24 to 36 kDa. Protein migrating as a band of molecular mass of about 45 kDa was identified as FOO. The second major band of about 60 kDa is ME (Figs.29, 30A, page 67). Antibodies against succinate thiokinase (STK), ferredoxin (Fdx), pyruvate:ferredoxin oxidoreductase (PFO) and Complex I subunits did not react with any of these hydrogenosomal proteins. As the faint bands are present only in parallels containing recombinant His-FOO, but absent in the parallel of pure hydrogenosomal lysate, these polypeptides probably are not of hydrogenosomal origin. I suppose that these proteins represent residual contaminants coming from bacterial lysate that FOO was purified from. The possibility that malic enzyme interacts directly with FOO is not probable. This protein is often co-purified with other hydrogenosomal proteins and its presence in all parallels containing hydrogenosomal lysate is likely of unspecific nature.

We hypothesized that FOO protein expression helps trichomonad cell to survive in conditions of oxidative stress. Therefore, TvFOOWT line overexpressing FOO was prepared, and hydrogenosomes isolated from this line were used in respiration experiments. We hypothesized that FOO overexpression might increase the rate of oxygen consumption by isolated hydrogenosomes.

Analysis of hydrogenosomal respiration was performed using Clark-type oxygen-monitoring electrode. NADH-dependent and pyruvate-dependent respirations were measured with hydrogenosomes from TvT1 and TvFOOWT cells and respiration rates were compared. Both NADH-dependent and pyruvate-dependent respiraton rates were

enhanced in TvFOOWT line in comparison with parent TvT1 trichomonads, with the exception of NADH-dependent respiration of hydrogenosomes isolated on 23.01.2006, that was not in accordance with other measurements. NADH-dependent respiration activity of TvFOOWT hydrogenosomes was decreased in these measurements.

In order to correlate the respiration rates of hydrogenosomes from TvFOOWT line with activities of major hydrogenosomal enzymes, I have determined the activities of four hydrogenosomal enzymes participating in redox reactions: malic enzyme, pyruvate: ferredoxin oxidoreductase, Complex I and hydrogenase. Comparison of these activities in TvFOOWT line with those of TvT1 line revealed enhanced activity of Complex I and pyruvate:ferredoxin oxidoreductase and reduced activity of malic enzyme in TvFOOWT cell line. These results corresponded at least in part to hydrogenosomal protein profiles on SDS-PAGE, showing relatively increased amount of PFO and reduced amount of ME in transformed trichomonads (Fig.32, page 69). As with respiration experiments, the enzymatic activities obtained by the measurement with hydrogenosomal samples isolated on 23.01.2006 did not fit into the determined trend.

Based on these results I assume that respiratory activity of transformed trichomonads is probably enhanced due to the increased activities of pyruvate:ferredoxin oxidoreductase and Complex I. This assumption is supported by the measurements with TvSUB 6 hydrogenosomal isolates. TvSUB6 hydrogenosomes were isolated from trichomonads transfected with MasterNEO plasmid carrying gene encoding for potential membrane subunit of protein complex homologous to mitochondrial Complex I (L. Bardoňová, Department of Parasitology, Charles University in Prague). These trichomonads probably do not express the introduced gene and were used as a control carrying MN plasmid without obvious phenotype. The changes in hydrogenosomal activities and respiration in TvSUB6 cell line were similar to those observed with TvFOOWT hydrogenosomes. Therefore, it appears that FOO overexpression phenotype, observed as changes in enzymatic activities and respiration rates of isolated hydrogenosomes, probably does not relate to FOO overexpression, but it is rather a secondary effect associated with trichomonad cell transfection or treatment with geneticin in the process of selection and cultivation of cells with acquired plasmid.

This issue should be addressed in future experiments, when only clonal cell lines should be used for transformation and determination of potential phenotype.

Since the trichomonads do not effectively express FOOHis and rec. His-FOO produced in bacteria might not be active, apart from being very difficult to keep in native

condition, I have decided to construct another expression system enabling purification of protein from trichomonad cell. I constructed a *T. vaginalis* expression vector named TagVag 3 with a *Strep*-tag sequence. The tag has high affinity to streptavidin, enabling simple purification of tagged protein. Gene encoding FOO was cloned into TagVag 3 and its expression was proven by immunodetection, but the activity of purified protein has not been determined yet. It is also possible to employ this construct in cross-linking method for detection of interacting proteins.

The level of FOO expression in metronidazole-sensitive strain TV 10-02 was compared to that of the resistant derivative MR 10-02 MR100, and was found to be lower in the hydrogenosomes of resistant cells. It is worth mentioning that in metronidazole-sensitive trichomonads the lowered affinity to oxygen was determined [72], but it is premature to speculate that decreased expression of FOO might lay behind this phenomenon. In metronidazole-resistant trichomonads, the expression and activities of many enzymes is altered, likely including also so far unidentified oxygen-reducing enzymes [5]. Nevertheless, the fact that metronidazole-resistant trichomonads are more sensitive to oxidative stress and have lower levels of a protein homologous to oxygen scavenging enzyme may not be a mere coincidence.

More recently, it has been shown that the flavodiiron proteins have a significant nitric oxide reductase activity [97,104]. It appears that NO is probably more preferred substrate than oxygen, but it may also be that these enzymes are bifunctional, participating both in oxidative and nitrosative stress prevention.

Reaction partners participating in NO reduction seem to be the same physiological partners as those participating in electrontransport to FDPs in reactions with oxygen. The product of NO reduction by FDPs have not been demonstrated yet, but it could be N_2O [97].

T. vaginalis may take benefit from such an enzymatic activity to counteract cytotoxic NO production by the host immune system.

It is necessary to isolate native FOO and to find its electrontransport partners for determination of FOO function in the hydrogenosome of *T. vaginalis*. For this purpose, the crosslinking experiments with FOO*Strep* and subsequent affinity purification of potential protein complex may prove useful.

7. Conclusions

In my master thesis project, I have studied the flavodiiron protein from *T. vaginalis* hydrogenosomes. This flavoprotein is homologous to recently defined group of flavodiiron proteins widespread among anaerobic and facultative anaerobic prokaryotes, and has been identified also in the genomes of some parasitic and anaerobic amitochondriate protozoa. In few cases where the activity of these proteins has been elucidated, it appears that they function as nitric oxide and oxygen reductases.

1. I have prepared *T. vaginalis* lines overexpressing FOO protein with different tag sequences (TvFOOHis, TvFOOHaHa, TvFOOStrep) and the line overexpressing the wild-type protein (TvFOOWT).

2. I have localized FOO protein in trichomonad cell. Using cell fractionation and immunofluorescence approach, I have determined that FOO is a hydrogenosomal protein.

3. I obtained recombinant His-FOO by affinity purification from bacterial lysate. Rec. His-FOO was used in determination of physical properties of the protein.

4. Flavin moiety of FOO protein was determined by TLC method as FMN.

5. Gel filtration chromatography determined that FOO is a protein with native molecular mass of about 92 kDa. Since the apparent molecular mass of the protein on denaturing SDS-PAGE is about 45 kDa, FOO is a dimer like its so far characterized homologues.

6. Respiration measurements revealed increased oxygen-reducing activity in hydrogenosomal isolates from TvFOOWT cell lines in comparison with TvT1 ones. Activities of hydrogenosomal enzymes PFO and Complex I were increased in TvFOOWT cells as well. In contrast, ME activity was decreased in TvFOOWT line in comparison with parent TvT1 trichomonads. Determination of these activities and respiration measurements with hydrogenosomes from the cell line harbouring

expression vector but without any apparent expression phenotype (TVSUB6) revealed changes similar to those observed in TvFOOWT line. Increased respiration rates and enzymatic activities probably do not relate to FOO overexpression, but rather represent a secondary effect of trichomonad cell transfection and selection following treatment with geneticin in the process of stabilization of the cell line carrying the expression plasmid.

7. Affinity purified rec. His-FOO is unstable and its use in activity determinations is compromised by high imidazole content.

8. The level of expression of FOO is decreased in metronidazole-resistant trichomonads.

Bottom line: In future experiments, it is necessary to determine the NO reductase activity of trichomonads overexpressing FOO and to identify the electron donor of FOO, before its function could be established.

8. References

[1] Petrin, D., Delgaty, K., Bhatt, R., and Garber, G. (1998) *Clin. Microbiol. Rev.* **11**, 300-317

[2] World Health Organisation (2001) Global Prevalence and Incidence of Selected Curable Sexually Transmitted Disease: Overview and Estimates, WHO/CDS/CSR/EDC/2001.10

[3] Sorvillo, F., Smith, L., Kerndt, P., and Ash, L. (2001) *Emerg. Infect. Dis.* **7**, 927-932

[4] Cotch, M. F. et al. (1997) *Sex Transm.dis.* **24**, 353-360

[5] Kulda, J. (1999*) Int. J. Parasitol.* **29**, 199-212

[6] Edwars, D. I. (1993) *J. Antimicrob. Chemother.* **31**, 9-20

[7] Yarlett, N., Lindmark, D.G., Golgberg, B., Moharami, M. A. and Bacchi, C. J. (1994) *J. Eukaryot. Microbiol.*, **41**(6), 554-9

[8] Sogin, M. L. (1997) *Curr. Opin. Genet. Dev.* **7**, 792-799

[9] Baldauf, S. L., Roger, A. J., Wenk-Seifert, I., and Doolittle, W. F. (2000) *Science* **290**, 972-977

[10] Embley, T. L., and Hirt, R. P. (1998) *Curr. Opin. Genet. Dev.* **8**, 624-629

[11] Roger, A. J. (1999) *Am. Nat.* **154**, S146-S163

[12] Dyall, S. D., and Johnson, P. J. (2000) *Curr. Opin. Microbiol.* **3**, 404-411

[13] Van der Giezen, M., Slotboom, D. J., Horner, D. S. Dyal, P. L., Harding, M., Xue, G. P., Embley, T. L., and Kunji, E. R. (2002) *EMBO J.* **21**, 572-579

[14] Honinberg, B.M. and Brugerrole, G. (1989) Structure in Trichomonads parasitic in humans. Ed. Honigberg, New York, Springer-Verlag, 5-35

[15] Brugerolle, G. (1991) *Protoplasma* **164**, 70–90

[16] Brugerolle, G. (1975-76) Cytologie ultrastructurale, systématique et évolution des Trichomonadida. Ann. Station. Bol. Besse-en-Chandesse 10,1-57

[17] Drmota, T., and Král, J. (1997) *Europ. J. Protistol.* **56**, 286-291

[18] Samuels, R. (1980) *J. Protozool.* **27**, 39A

[19] Brugerolle, G. (1975) *Protistologica* **11**, 457-468

[20] Lindmark, D. G., Müller, M. (1973) *J. Biol. Chem.* **248**, 7724-7728

[21] Brugerolle, G. (1972) *Protistologica* **8**,353-363

[22] Benchimol, M., M. E. A. Pereira, C. A. Elias, W. De Souza (1981) *J. Protozool.* **28**, 337-341

[23] Kulda, J. and Kunzová, E. (1987) Standardní metody diagnostiky trichomonózy. *Acta Hygien. Epidem. Microbiol.*, príloha č.16/1987, 26

[24] Lindmark, D. G., Müller, M. (1974*b*) *J. Protozool.* **21**, 374-378

[25] Lindmark, D. G., Müller, M., and Shio, H. (1975) *J. Parasitol.* **61**, 552-554

[26] Müller, M. (1980) *Symp. Soc. Gen. Microbiol.* **30**, 127-142

[27] Alderete, J. F. (1983) *Infect. Immun.* **39**, 1041-1047

[28] Nielsen, M. N., Diemer, N. H. (1976) *Cell Tissue Res.* **167**, 461-465

[29] Honigberg, B. M., Volkmann, D., Entzeroth, R., Scholtyseck, E. (1984) *J. Protozool.* **31**, 116-131

[30] Benchimol, M., De Souza, W. (1983) *J. Protozool.* **30**, 422-425

[31] Benchimol, M., Johnson, P. J., De Souza, W. (1996) *Biol. Cell* **87**, 197-205

[32] Berkaloff, A., Bourguet, J., Favard, P., Lacroix, J. C. (1978) Biologie et Physiologie Cellulaires. Vol II, Hermann, Paris

[33] Larsen, W. J. (1970) *J. Cell Biol.* **47**,373-383

[34] Tandler, B., Hoppel, L. (1973) *J. Cell Biol.* **56**, 266-272

[35] Turner, G., Müller, M. (1983) *J. Parasitol.* **69**, 234-236

[36] Wang, A. L., and Wang, C. C. (1985) *Mol. Biochem. Parasitol.* **14**, 323-335

[37] Clemens, D. L., Johnson, P. J. (2000) *Mol. Biochem. Parasitol.* **106**(2),307-13

[38] Lahti, C. J., Johnson, P. J. (1991) *Mol. Biochem. Parasitol.* **46**, 307-310

[39] Johnson, P. J., Lahti, C. J. Bradley, P. J. (1993) *J. Parasitol* **79**, 664-670

[40] Pütz, S., Gelius-Dietrich, G., Piotrowski, M., Henze, K. (2005) *Mol. Biochem. Parasitol.* **142**(2), 212-23

[41] Johnson, P. J., Bradley, P. J. and Lahti, C. J. (1995) Cell Biology of Trichomonads: Protein targeting to the hydrogenosome. In Boothroyd, J. C. and Komuniecki, R. (eds). *Molecular Approaches to Parasitology.* Wiley-Liss, New York. pp. 399-411

[42] Bradley, P. J., Lahti,C. J., Plümper, E. and Johnson, P. J. (1997) *EMBO J.* **16**, 3484-3493

[43] Johnson, P. J., D' Oliveira, C. E., Gorrel, T. E. and Müller, M. (1990) *Proc. Natl. Acad. Sci. USA* **87**, 6097-6101

[44] Von Heijne, G., Steppuhn, J., Herrmann, R. G. (1989) *Eur. J. Biochem.* **180**, 535-545

[45] Plümper, E., Bradley, P. J. and Johnson, P. J. (2000) *Mol. Biochem. Parasitol.* 106, 11-20

[46] Fenchel, T. and Finlay, B. J. (1991 a) *Eur. J. Protistol.* **26**, 201-215

[47] Finlay, B. J. and Fenchel, T. (1989) *FEMS Microbiol. Lett.* **65**, 311-314

[48] Van Bruggen, J. J. A., Stumm, C. K. and Vogels, G. D. (1983) *Arch. Microbiol.* **136**, 89-95

[49] Yarlett, N., Hann, A. C., Lloyd, D. and Williams, A. G. (1981) *Biochem. J.* **200**, 365-372

[50] Yarlett, N., Hann, A. C., Lloyd, D. and Williams, A. G. (1983) *Comp. Biochem. Physiol.* **74B**, 357-364

[51] Yarlett, N., Coleman, G. S., Williams, A. G. and. Lloyd, D. (1984) *FEMS Microbiol. Lett.* **21**, 15-19

[52] Snyers, L., Hellings, P., Bovy-Kesler, C. and Thines-Sempoux, D. (1982) *FEBS Lett.***137**, 35-39

[53] Paul, R. G., Williams, A. G. and Butler, R. D. (1990) *J. Gen. Microbiol.* **136**, 388-396

[54] Yarlett, N., Orpin, C. G., Munn, E. A., Yarlett, N. C. and Greenwood, C. A. (1986*a*) *Biochem. J.* **236**, 729-739

[55] Marvin-Sikkema, F. D., Lahpor, G. A., Kraak, M. N., Gottschal, J. C. and Prins, R. A. (1992) *J. Gen. Microbiol.***138**, 2235-2241

[56] Müller, M. (1993) *J. Gen. Microbiol.* **139**, 2879-2889

[57] Hrdý, I. and Müller, M. (1995*b*) J. Euk. Microbiol. **42**, 593-603

[58] Drmota, T., Proost, P., Van Ranst, M., Weyda, F., Kulda, J. and Tachezy, J. (1996) *Mol. Biochem. Parasitol.* **83**, 221-234

[59] Williams, K., Lowe, P. N. and Leadlay, P. F. (1987) *Biochem. J.* **246**, 529-536

[60] Hrdý, I. and Müller, M. (1995*a*) *J. Mol. Evol.* **41**, 388-396

[61] Jenkins, T. M., Gorrell, T. E., Müller, M. and Weitzman, P. D. J. (1991) *Biochem. Biophys. Res. Commun.* **179**, 892-896

[62] Müller, M. (1988) *Ann. Rev. Microbiol.* **42**, 465-488

[63] Gorrell, T. E., Yarlett, N. and Müller, M. (1984) *Carlsberg Res. Commun.* **49**, 259-268

[64] Vidakovic, M. S., Franckiewicz, G. and Germanas, J. P. (1996) *J. Biol. Chem.* **271** (25), 14734-14739

[65] Coombs, G. and Müller, M. (1995) In: Biochemistry and molecular biology of parasites. Eds Marr, M. and Müller, M. Academic Press Ltd., pp. 33-47

[66] Steinbuchel, A. and Müller, M.. (1986) *Mol. Biochem. Parasitol.* **20**, 57–65

[67] Rasoloson, D., Vanacova, S., Tomkova, E., Razga, J., Hrdy, I., Tachezy, J., Kulda, J. (2002) *Microbiology* **148**, 2467–2477

[68] Payne, M.J., Chapman, A. and Cammack, R. (1993) *FEBS Lett.* **317**, 101-104

[69] Meyer, J. and Gagnon, J. (1991) *Biochemistry* **30** (40), 9697-9704

[70] Yagi, T., Kimura, K. and Inokuchi, H. (1985) *J. Biochem.* **97** (1), 181-187

[71] Čerkasov, J., Čerkasovová, A. and Kulda, J. (1978) *J. Biol. Chem.* **253**, 1207-1214

[72] Yarlett, N., Yarlett, N. C. and Lloyd, D. (1986*b*) *Mol. Biochem. Prasitol.* **19**, 111-116

[73] Müller, M. (1973) *J. Cell Biol.* **57**, 453-474

[74] Lloyd, D., Lindmark, D. G. and Müller, M. (1979*b*) *J. Parasitol.* **65**, 466-469

[75] Lloyd, D., Lindmark, D. G. and Müller, M. (1979*a*) *J. Gen. Microbiol.* **115**, 301-307

[76] Lloyd, D. and Kristensen, B. (1985) *J. Gen. Microbiol.* **131**, 849-853

[77] Müller, S., Liebau, E., Walter, R. D. and Krauth-Siegel, R. L., (2003) *Trends Parasitol.* **1 9**, 320-328

[78] Ellis, J. E., Yarlett, N., Cole, D., Humphreys, M. J., and Lloyd, D. (1994) *Microbiology* **140**, 2489-2494

[79] McKie, A. E., Edlind, T., Walker, J., Mottram, J. C., and Coombs, G. H. (1998*) J. Biol. Chem.* **273**, 5549-5556

[80] Chae, H. Z., Chung, S. J., and Rhee, S. G., (1994) *J. Biol. Chem.* **269**, 27670-27678

[81] Netto, L. E. S., Chae, H. Z., Kang, S. W., Rhee, S. G. and Stadtman, E. R. (1996) *J. Biol. Chem.* **271**, 15315-15321

[82] Rhee, S. G., Kang, S. W., Netto, L. E. S., Seo, M. S. and Stadtman, E. R. (1999) *Biofactors***10**, 207-226

[83] Chae, H. Z., Kang, S. W. and Rhee, S. G. (1999) *Methods. Enzymol.* **300**, 219-226

[84] Park, S. G., Cha, M. K., Jeong, W. and Kim, I. H. (2000) *J. Biol. Chem.* **275**, 5723-5732

[85] Mouaheb, N., Thomas, D., Verdoucq, L., Monfort, P., and Meyer, Y. (1998) *Proc. Natl. Acad. Sci. USA* **95**, 3312-3317

[86] Hofmann, B., Hecht, H. J. and Flohe, L. (2002) *Biol. Chem.* **383**, 347-364

[87] Coombs, G. H., Westrop, G. D., Suchan, P., Puzova, G., Hirt, R. P., Embley, T. M., Mottram, J. C. and Müller, S. (2004) *J. Biol. Chem.* **279**, 5249-5256

[88] Wasserfallen, A., Ragettli, S., Jounaneu, Y. and Leisinger, T. (1998) *Eur. J. Biochem.* **254**, 325-332

[89] Frazao, C., Silva, G., Gomes, C. M., Matias, P., Coelho, R.,Sieker, L., Macedo, S., Liu, M.Y., Oliviera, S., Texeira, M., Xavier, A.V., Rodrigues-Pousada, C., Carrondo, M.A. and Le Gall, J. (2000) *Nat. Struct. Biol.* **7**, 1041-1045

[90] Chen, L., Liu, M.Y., LeGall, J., Fareleira, P., Santos, H. and Xavier, A.V. (1993*b*) *Biochem. Biophys. Res. Commun.* **193**, 100-105

[91] Gomes, C. M., Silva, G., Oliviera, S., LeGall, J., Liu, M.Y., Xavier, A.V., Rodrigues-Pousada, C. and Texeira, M. (1997) *J. Biol. Chem.* **272**, 22502-22508

[92] Okinaka, Y., Yang, C. H., Perna, N. T. and Keen, N. T. (2002) *Mol. Plant. Microbe Interact.* **15**, 619-629

[93] Saraiva, L. M., Vicente, J. B. and Texeira, M. (2004) *Adv. Microb. Physiol.* **49**, 77-129

[94] Chen, L., Liu, M.Y., LeGall, J., Fareleira, P., Santos, H. and Xavier, A.V. (1993*a*) *Eur. J. Biochem.* **216**, 443-448

[95] Jenney, F. E. J., Verhagen, M. F., Cui, X. and Adams, M. W. (1999) *Science* **286**, 306-309

[96] Das, A., Coulter, E. D., Kurtz, D.M., Jr. and Ljungdahl, L. G. (2001) *J. Bacteriol.* **183**, 1560-1567

[97] Silaghi-Dumitrescu, R., Coulter, E. D., Das, A., Ljungdahl, L. G., Jameson, G. N., Huynh, B. H. and Kurtz, D. M., Jr. (2003) *Biochemistry* **42**, 2806-2815

[98] Gomes, C. M., Vicente, J. B., Wasserfallen, A. and Texeira, M. (2000) *Biochemistry* **39**, 16230-16237

[99] Vicente, J. B., Gomes, C.M., Wasserfallen , A and Texeira, M. (2002) *Biochem. Biophys. Res. Commun.* **294**, 82-87

[100] Kaneko, T., Sato, S., Kotani, H., Tanaka, A., Asamizu, E., Nakamura, Y., Miyajima, N., Hirosawa, M., Sugiura, M., Sasamoto, S., Kimura, T., Hosouchi, T., Matsuno, A., Muraki, A., Nakazaki, N., Naruo, K., Okumura, S., Shimpo, S, Takeuchi, C., Wada, T., Watanabe, A., Yamada, M., Yasuda, M. and Tabata, S. (1996) *DNA Res.* **3**, 109-136

[101] Nakamura, Y., Kaneko, T., Sato, S. Ikeuchi, M., Katoh, H., Sasamoto, S., Watanabe, A., Iriguchi, M., Kawashima, K., Kimura, T., Kishida, Y., Kiyokawa,

C., Kohara, M., Matsumoto, M., Matsuno, A., Nakazaki, N., Shimpo, S., Sugimoto, M., Takeuchi, C., Yamada, M. and Tabata, S. (2002) *DNA Res.* **9**, 123-130

[102] Gardner, P. R., Constantino, G. and Salzman, A. L. (1998*a*) *J. Biol. Chem.* **273**, 26528-26533

[103] Gardner, A. M., Helmick, R. A. and Gardner, P. R. (2002) *J. Biol. Chem.* **277**, 8172-8177

[104] Gomes, C. M., Giuffre, A., Forte, E., Vicente, J. B., Saraiva, L. M., Brunori, M. and Texeira, M. (2002b) *J. Biol. Chem.* **277**, 25273-25276

[105] Brunori, M., Giuffre, A., Sarti, P., Stubauer, G. and Wilson, M. T. (1999) *Cell. Mol. Life Sci.* **56**, 549-557

[106] Brunori, M. (2001) *Trends Biochem. Sci.* **26**, 21-23

[107] Forte, E., Urbani, A., Saraste, M., Sarti, P., Brunori, M. and Giuffre, A. (2001) *Eur. J. Biochem.* **268**, 6486-6491

[108] Sarti, P., Giuffre, A., Barone, M. C., Forte, E., Mastronicola, D. and Brunori, M. (2003) *Free Radic. Biol. Med.* **34**, 509-520

[109] Hrdý, I.., Hirt, R. P., Doležal, P., Bartoňová, L., Foster, P. G., Tachezy, J., Embley, T. M. (2004) *Nature* 432,618-22

[110] Diamond, L. S. (1957) *J. Parasitol.* **43**, 488-490

[111] Laemmli, U. K. (1970) *Nature* **227**, 680-685

[112] Lowry, O. H., Rosenburg, N. J., Farr, A. L. and Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265-275

Svoluji k zapůjčení této diplomové práce pro studijní účely a prosím, aby byla vedena evidence vypůjčovatelů.

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