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Naegleria fowleri: A free-living highly pathogenic amoeba contains trypanothione/trypanothione reductase and glutathione/glutathione reductase systems

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Abstract

This paper presents definitive data showing that the thiol-bimane compound isolated and purified by HPLC from *Naegleria fowleri* trophozoites unequivocally corresponds by matrix assisted laser-desorption ionization-time-of-flight MS, to the characteristic monoprotonated ion of trypanothione-(bimane)$_2$ [M^+H^+] of *m/z* 1104.57 and to the trypanothione-(bimane) of *m/z* 914.46. The trypanothione disulfide $T(S)_2$ was also found to have a molecular ion of *m/z* 723.37. Additionally HPLC demonstrated that thiol-bimane compounds corresponding to cysteine and glutathione were present in *Naegleria*. The ion patterns of the thiol-bimane compounds prepared from commercial trypanothione standard, *Entamoeba histolytica* and *Crithidia luciliae* are identical to the *Naegleria* thiol-bimane compound. Partially purified extracts from *N. fowleri* showed the coexistence of glutathione and trypanothione reductases activities. There is not doubt that the thiol compound trypanothione, which was previously thought to occur only in Kinetoplastida, is also present in the human pathogens *E. histolytica* and *N. fowleri*, as well as in the non-pathogenic euglenozoan *E. gracilis*. The presence of the trypanothione/trypanothione reductase system in *N. fowleri* creates the possibility of using this enzyme as a new "drug target" for rationally designed drugs to eliminate the parasite, without affecting the human host.

Index Descriptors and Abbreviations: Drug target; *Naegleria fowleri*; Cysteine; Glutathione; Trypanothione; Glutathione reductase; Trypanothione reductase; $T(S)_2$, trypanothione disulphide; Gspd, glutathionyl-spermidine; MALDI-TOF, matrix assisted laser-desorption ionization-time-of-flight; mBBr, monobromobimane

1. Introduction

Naegleria fowleri is a free-living amoeba that can be pathogenic to humans, producing a primary acute meningoencephalitis that causes death of the host within a few days of the first symptoms being detected. Normally, it is found in lakes, ponds, swimming pools, and waste water sewage. The infection route begins with transmission via the nasal mucosa and olfactory nerves, crossing the cribriform plate, and gaining access to the central nervous system. Once in the brain, extensive inflammation, hemorrhage, and necrosis occurs leading to death within 3 to 7 days (John, 1993).

From a metabolic point of view, *N. fowleri* is an aerobic organism with mitochondria that have the capacity to synthesize glutathione and to promote glutathione reductase activity. Thiol compounds like glutathione and its reducing enzyme glutathione reductase, permit the majority of eukaryotes and prokaryotes to maintain a high thiol/disulphide ratio, providing protection against oxygen toxicity. However, there are examples of bacteria that do not produce glutathione (Fahey et al., 1978). Instead, these

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synthesize other low-molecular-mass thiol compounds like glutathione amide and its perthiol in anaerobic sulfur bacteria (Bartsch et al., 1996) and mycothiol in most actinomycetes (Newton et al., 1996).

Some human pathogenic protozoa also use other thiol compounds of their own, which have specific enzymes not present in the host. Examples already exist of these pathogenic protozoa, such as trypanothione (bis glutathionyl-spermidine) with its enzyme trypanothione reductase, originally identified in trypanosomatids (Fairlamb et al., 1985; Shames et al., 1986).

Although the thiol compound trypanothione and its reducing enzyme trypanothione reductase (TR) were previously supposed to occur only in trypanosomatids (Fairlamb et al., 1985; Shames et al., 1986) it has been shown that TR is not unique to these parasites, since Montrichard et al., 1999 demonstrated the coexistence of both TR and glutathione reductase (GR) in the photosynthetic non-trypanosomatid euglenozoan.

Moreover, previously a gene for the TR enzyme obtained from *E. histolytica* by PCR amplification of its DNA has been described, as has the presence of a NADPH-dependent TR activity in vitro with partially purified extracts (Tamayo et al., 2005). Using MALDI-TOF MS, it was also shown that the thiol-bimane compound isolated and purified from *E. histolytica* trophozoites (Ondarza et al., 2005) corresponds by MALDI-TOF MS to the characteristic monoprotonated ion of trypanothione-(bimane)$_2$.

This paper presents definitive MALDI-TOF MS data to show that a thiol-bimane compound, previously named compound V isolated and purified from *N. fowleri* trophozoites (Ondarza et al., 2003), corresponds to the characteristic monoprotonated ion of trypanothione-(bimane)$_2$ with an *m*/*z* of 1104.57 and that partially purified extracts from *Naegleria* contain clear NADPH-dependent glutathione and trypanothione reductase activities.

2. Materials and methods

2.1. Reagents

Thiolyte (monobromobimane; mBBr) was obtained from Calbiochem and oxidized trypanothione T(S)$_2$ were from Bachem (Torrance, CA, USA). L-Cysteine was from E. Merck (Darmstadt, Germany), water-grade HPLC was from Burdick and Jackson, dithiothreitol (DTT) was from Gibco, and glutathione and *N*-ethylmaleimide (NEM) were from Sigma.

2.2. Cell culture conditions

Highly pathogenic *N. fowleri* ATCC 30808 (kindly provided by Dr. Mineko Shibayama, Centro de Investigaciòn y de Estudios Avanzados, Instituto Politécnico Nacional, México D.F., México) was grown axenically at 37 °C for 60 h in culture medium containing bacto casitone, 2% pancreatic digest of casein, and 10% inactivated fetal bovine serum. The total number of trophozoites present in 40 ml of normal culture medium, harvested at the 60 h was approx. 25.4×10^6. The pellet obtained was around 40–60 mg (wet weight) at this stage.

Crithidia luciliae epimastigotes (kindly supplied by Dr. Victor Monteón Padilla, Instituto Nacional de Cardiología, México, D.F., México) were grown at 27 °C in flasks containing 100 ml of saline culture medium with 3.7% (w/v) brain/heart infusion (Oxoid), 3% (v/v) fetal bovine serum, 1% (w/v) dextrose, and 2.3% (w/v) agar, and harvested after 60 h.

Escherichia coli (ATCC-25922 and DH5-α strains) was grown in 5 ml LB liquid medium, pH 7.0, at 37 °C overnight.

2.3. Analysis of low-molecular-mass thiol compounds

The reduced thiol compounds from 40 ml of *N. fowleri* cultured for 60 h were analyzed by fluorescent labeling derivatization with acetonitrile and 2 mM mBBr (pH 8.0) for 20 min at 60 °C and separated by HPLC using a Lichrosphere RP C-18.5 μm pore-sized column (4 mm × 250 mm) operated at 30 °C at a flow rate of 1.2 ml/min.

The results were obtained using 20 μl of derivatized extract, which was injected into a model 1100 Hewlett—Packard HPLC equipped with a fluorescent detector. An elution gradient of methanol/acetic acid/water was used as follows: buffer A, aq. 0.25% acetic acid titrated to pH 3.5; buffer B, methanol. The elution program with the linear gradient was as follows: 0 min, 15% B; 20 min, 23% B; 25 min, 35.5% B; 45 min, 40% B; 50 min, 60% B; re-injection.

The thiol-bimane compounds were detected by comparing against commercial standards of cysteine, glutathione, Gspd, and trypanothione derivatives with bimane. They were shown to be thiols since all of them were blocked by reaction with 5 mM *N*-ethylmaleimide at 60 °C for 20 min before the formation of the bimane derivatives. In this way, thiols could also be differentiated from the reagent peaks when analyzed by HPLC.

For electrolytic reduction, the technique described by Saetre and Rabenstein (1978) was followed.

2.4. Mass spectrometry by ESI and MALDI-TOF analyses

Some of the preliminary ESI and MALDI-TOF analyses were performed by The Scripps Research Institute (La Jolla, CA, USA) and the Center for Marine Biotechnology and Biomedicine, Scripps Institution of Oceanography, UCSD (University of California at San Diego), La Jolla, CA, USA. Definitive MALDI-TOF results here described were performed in the laboratory at Cuernavaca, Morelos, Mexico, using the Voyager-DE PRO equipment from Applied Biosystems, INC.

2.5. Enzyme assays

Partial purification of glutathione reductase and trypanothione reductase from *N. fowleri*, *C. luciliae*, and *E. coli*