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Leishmania infantum trypanothione reductase is a promiscuous enzyme carrying an NADPH:O_2 oxidoreductase activity shared by glutathione reductase

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ABSTRACT

Background: Leishmania infantum is a protozoan of the trypanosomatid family causing visceral leishmaniasis. *Leishmania* parasites are transmitted by the bite of phlebotomine sand flies to the human host and are phagocyted by macrophages. The parasites synthesize N1-N8-bis(glutationyl)-spermidine (trypanothione, TS_2), which furnishes electrons to the tryparedoxin-tryparedoxin peroxidase couple to reduce the reactive oxygen species produced by macrophages. Trypanothione is kept reduced by trypanothione reductase (TR), a FAD-containing enzyme essential for parasite survival.
Methods: The enzymatic activity has been studied by stopped-flow, absorption spectroscopy, and amperometric measurements.
Results: The study reported here demonstrates that the steady-state parameters change as a function of the order of substrates addition to the TR-containing solution. In particular, when the reaction is carried out by adding NADPH to a solution containing the enzyme and trypanothione, the K_M for NADPH decreases six times compared to the value obtained by adding TS_2 as last reagent to start the reaction (1.9 *vs.* 12 μM). More importantly, we demonstrate that TR is able to catalyze the oxidation of NADPH also in the absence of trypanothione. Thus, TR catalyzes the reduction of O_2 to water through the sequential formation of C(4a)-(hydro)peroxyflavin and sulfenic acid intermediates. This NADPH:O_2 oxidoreductase activity is shared by *Saccharomyces cerevisiae* glutathione reductase (GR).
Conclusions: TR and GR, in the absence of their physiological substrates, may catalyze the electron transfer reaction from NADPH to molecular oxygen to yield water.
General significance: TR and GR are promiscuous enzymes.

1. Introduction

Leishmaniasis is a neglected disease that affects several million people worldwide with a high mortality rate [1–4]. It is caused by the *Leishmania* species, which are human protozoan parasites of the Trypanosomatidae family. Trypanosomatids differ from other eukaryotes in their specific redox metabolism since the eukaryotic glutathione/glutathione reductase system is replaced by the unique trypanothione/trypanothione reductase (TS_2/TR) system [5–7]. Trypanothione (N1,N8-bis(glutathionyl)spermidine) is a low-molecular-weight dithiol in which two glutathione moieties are covalently tethered together by a spermidine spacer *via* two amide bonds, with the C-terminal glycine residues of glutathione and the terminal amino groups of spermidine. This molecule is synthesized by trypanothione synthetase (TryS), and its reduced form ($T(SH)_2$) is used by the couple tryparedoxin/tryparedoxin peroxidase I (TXN/TXNPx) to neutralize the hydrogen peroxide produced by the macrophages during the infection [8,9].

TR, which reduces TS_2, is essential for parasite survival and its inhibition was shown to highly increase the intracellular levels of reactive oxygen species lethal for the parasites [10,11]. For this reason, TR is an interesting target for parasite-specific drug development against diseases caused by trypanosomatids [12,13].

The structure of TR from *Leishmania (L.) infantum* has been solved in both oxidized and reduced forms [14,15]. TR is a functional homodimer

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containing three structural domains: (i) a NADPH-binding domain; (ii) a FAD-binding domain, containing on the *Si* side of the FAD group, two redox-reactive cysteine residues (52 and 57 in *L. infantum* numbering); and (iii) a trypanothione-binding domain located at the monomer–monomer interface, placed on the FAD *Si* side of each FAD-binding domain. The reaction catalyzed by TR is the transfer of two electrons from NADPH to oxidized trypanothione ($T(S)_2$) to yield $NADP^+$ and reduced trypanothione ($T(SH)_2$) according to the following reaction:

$$NADPH + T(S)_2 + H^+ \rightarrow NADP^+ + T(SH)_2.$$

In the TR reaction, binding of NADPH and electron transfer to the FAD group generates a charge-transfer complex with a typical broad low extinction spectroscopic signature in the 530 nm region of the visible spectrum, characterized by a resonant charged thiol-FAD complex. Subsequent binding of $T(S)_2$ to the interface domain results in electron transfer to yield $T(SH)_2$ and the regenerated enzyme, ready for an additional turnover cycle, provided sufficient NADPH and oxidized trypanothione are available. Most experimental published protocols [16,17] for the determination of the steady-state activity and parameters of TR involve addition of TR and NADPH to a buffered solution and starting the reaction by addition of the $T(S)_2$ substrate. The time course is followed at 340 nm representative of the oxidation state of NADPH.

The K_M and the k_{CAT} for both NADPH and $T(S)_2$ have been determined with this protocol for *L. infantum*, *L. donovani*, *Trypanosoma* (*T.*) *cruzi*, *T. congolense*, *T. brucei*, and *Crithidia* (*C.*) *fasciculata* TR [15,17–26]. In particular, the measured K_M of *Leishmania* species TR for $T(S)_2$, ranges from 36 to 72 μM, whereas the measured K_M for NADPH ranges from 9 to 12 μM. In this paper, we show that the use of the traditional protocol, where the reaction is started by adding TS_2 to the reaction mixture containing TR and NADPH, introduces systematic errors for the calculation of steady-state parameters. The order of addition of substrates determines the time-dependent kinetic profile of the TR reaction. Moreover, the experimental data reported here show that TR displays substantial promiscuity since in the absence of the $T(S)_2$ substrate, and in the presence of O_2 (but not when the system is anaerobic), TR is active in the oxidation of NADPH to yield $NADP^+$ and H_2O. Spectroscopic evidence together with amperometric experiments suggest that TR displays an additional O_2-reductase enzymatic activity, which is insensitive to catalase. Moreover, the spectroscopic data reported here clearly show that this promiscuous oxidase activity is displayed also by the glutathione reductase (GR) from *Saccharomyces cerevisiae*, and for this reason it can be considered a general property of the glutathione reductase family.

2. Materials and methods

2.1. Materials

Cloning, expression, and purification of TR from *Leishmania infantum* was carried out as already described [14,15]. The average FAD-to-protein ratio was 0.9. NADPH (Sigma Aldrich) and $T(S)_2$ (Bachem) were purchased and used as such. Concentrated TR (100–500 μM) was dissolved in 50 mM Hepes (4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid) buffer pH 7.5 containing 40 mM sodium chloride (hereafter referred to as buffer). This buffering system was used in all experiments. Glutathione reductase from *S. cerevisiae* (*Sc*GR) was purchased from Sigma–Aldrich as ammonium sulfate suspension. The protein was thoroughly dialyzed against 50 mM Hepes buffer, containing 40 mM sodium chloride at pH 7.5.

2.2. Stopped-flow experiments

Stopped-flow experiments were carried out on a Applied Photophysics thermostated stopped-flow apparatus (Leatherhead, UK) equipped with a Xe lamp, with input and output monochromator slits of 1 mm, and with a dead-time 1.3 ms determined by using the CO binding reaction to myoglobin. All the experiments have been carried out at 20 °C.

2.2.1. Determination of the steady-state parameters of Leishmania infantum TR in the NADPH oxidation reaction

In the stopped-flow experiment of Fig. 1, 10 nM TR dissolved in buffer was introduced in the stopped-flow syringe to which 100 μM $T(S)_2$ was added. This solution was rapidly mixed with NADPH at increasing concentrations, ranging from 0.92 to 18.2 μM. NADPH oxidation was followed at 340 nm, and the complete time profiles recorded. Initial rates were determined by linear regression of the first 2% of the data points of the reaction, and converted to μM/s units by using the molar extinction coefficient of 6220 M^{-1} cm^{-1} [27].

2.2.2. Kinetics of NADPH oxidation by TR and effect of substrate order addition

The kinetics of NADPH oxidation by TR has been performed in two ways by changing the order of substrate addition. In the first experiment, 1 μM TR premixed with 100 μM $T(S)_2$ in one syringe was mixed with 26 μM NADPH inserted in the second syringe, and NADPH oxidation followed at 340 nm. In the second experiment, 1 μM TR premixed with 26 μM NADPH in one syringe was mixed with 26 μM $T(S)_2$ inserted in the second syringe, and the traces have been measured at 340 nm and at different times from the premixing procedure.

2.3. Amperometric O_2 measurements

Amperometric O_2 measurements were performed using a high-resolution respirometer (Oxygraph-2 k, Oroboros Instruments) with a 1.5 ml gas-tight thermostated chamber. Temperature of all measurements was 20 °C. The O_2 concentration was measured directly. 20 μM TR was added to the amperometic 1.5-ml thermostated cell (20 °C) at time zero. Subsequently, increasing amounts of NADPH were added corresponding to 5 and 20 μM NADPH. Overall 50 μM NADPH was added, which is in excess over the amount of TR present. At the end of the experiment, catalase (2 ng/ml) was added to monitor the possible formation of hydrogen peroxide during the reaction.

2.4. Time resolved UV-visible spectra

UV-visible spectra were recorded on a thermostated Jasco V-650 spectrophotometer or a Hewlett Packard 8453 photodiode array

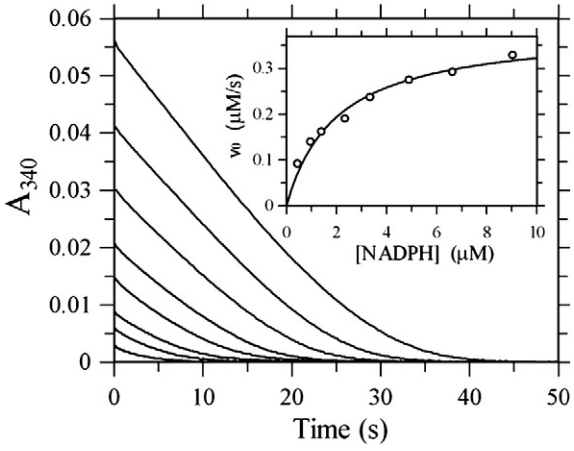

Fig. 1. Determination of the steady-state parameters of *Leishmania infantum* TR by stopped-flow spectroscopy. Inset: plot of the initial rates of NADPH oxidation by TR as a function of NADPH concentration (after mixing concentrations are given in the abscissa). The solid line was obtained by non-linear regression of the data points to the Michaelis–Menten equation.