Vanadate as a Futile, Superoxide Ion-producing Substrate of Trypanothione Reductase from *Trypanosoma cruzi*

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Recombinant *Trypanosoma cruzi* trypanothione reductase can oxidize NADPH in a futile cycle in the presence of vanadate ion as a result of the generation of reactive oxygen and other radical species from the active site dithiol and the NADPH. This process is inhibited by competitive inhibitors of trypanothione reductase catalyzed reduction of trypanothione disulfide.

Major parasitic diseases, including African Sleeping Sickness, Chagas' disease, and Leishmaniasis are caused by pathogenic parasites of *Trypanosoma spp.* and *Leishmania spp.* Infection can be fatal and many millions are at risk. The currently available drugs are few, and inadequate. In mammals potential redox damage faces the glutathione (GSH)-based system, during the action of which glutathione disulfide is formed. Regeneration of protective GSH from GSSG is catalyzed by glutathione reductase (GR). In the parasites there is no GR, but there is an analogous enzyme, trypanothione reductase (TR). TR was soon proposed as a target for the rational design of anti-trypanosomal drugs.[1–3] Inhibitors have now been designed by rational drug design approaches against TR (reviews[4,5]). Nitrofurans and quinones inhibit TR[6–8] and can act as subversive substrates[6] producing reactive oxygen species, including superoxide ion.

Vanadate V(V) reduction to V(IV) has been reported for thiols and other reductants.[9] Superoxide ion plus V(V) initiate a free radical chain oxidation of NAD(P)H.[10,11] Flavoenzyme dehydrogenases in general including GR can generate superoxide ion,[10,12,13] which has also been proposed as the basis of the V(V) stimulation by NAD(P)H oxidoreductases.[14] This is in contrast to these enzymes acting directly as vanadium(V) reductases:[15–17] a detailed review explains these disparate explanations.[13] Given the sensitivity of *T. cruzi* to oxidative stress and the similar chemical mechanisms of action of GR and TR, we tested recombinant TR from *T. cruzi* (isolated and assayed as described[18]) for vanadate-stimulated superoxide production.

A standard solution of ammonium vanadate (NH_4VO_4) in 1 M HCl (1 mg/mL, 19.6 mM of V[V]) was provided by Dr. Phil Day, University of Manchester. The solution was neutralized to pH 7 by NaOH and diluted to 10 mM prior to assay. Alternately solid ammonium metavanadate was dissolved in milliQ-water, made acidic (pH 4–5) using dilute HCl and the pH adjusted to 7 by dilute NaOH. Each such solution was prepared freshly before each use. The rate of NADPH oxidation was measured as a function of enzyme concentration, the reaction mixture (1 mL) contained vanadate ion (0.5 mM), NADPH (0.1 mM), and TR (2.5–25 μg) in 0.02 M HEPES pH 7.25 buffer. Little oxidation of NADPH was observed when NADPH was incubated with vanadate(V) alone in buffer. However, in the presence of TR, the rate NADPH oxidation increased linearly with TR concentration. The initial rate as a function of vanadate concentration (Figure 1) followed the Michaelis–Menten equation (apparent K_m and k_{cat} values 0.40 ± 0.07 mM and 84 ± 5 min^{-1}, respectively).

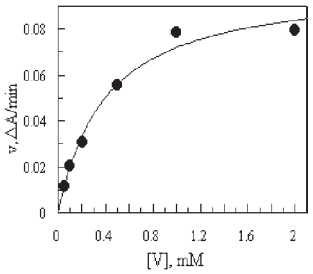

Figure 1. *T. cruzi* trypanothione reductase (20 μg/mL) reduction of vanadate ion (V) measured at 340 nm in 20 mM HEPES buffer pH 7.25 at 25 °C. Line is by nonlinear least squares regression fit to the Michaelis–Menten equation ($V_{max} = 0.1006 \pm 0.006$ ΔA·min^{-1}, $K_m = 0.40 \pm 0.07$ mM). The assay contained NADPH (0.1 mM), vanadate (0.05–2 mM), and buffer (20 mM Hepes) in a final volume of 1.00 mL. After incubation at 25 °C for 3 min, any background reaction was recorded and then reaction was initiated by addition of TR (20 μL 20 μg). The initial rate was calculated as ΔA·min^{-1}.

This vanadate-NADPH activity was inhibited by the presence of competitive inhibitors[18] of the trypanothione disulfide cleavage activity of TR, such as chlorpromazine. Under the conditions used the I_{50} value for chlorpromazine in the vanadate/NADPH reaction was 13 μM: using the apparent K_m value for vanadate, the calculated I_{50} value for chlorpromazine is 15 μM based on a K_i of 10 μM.[18] The inhibition observed in the vanadate reaction is most simply explained if some of the components of the active site of TR are required for the vanadate reaction. It does not necessarily indicate that vanadate reacts at the active site.

The stoichiometry of the reaction was determined using vanadate (0.05 mM), TR (40 μg·mL^{-1}) and NADPH (0.05–2 mM). When the concentration of vanadate ion and TR were kept constant and that of NADPH varied, the amount of the NADPH oxidation was related to its concentration. Several molar equivalents of NADPH were consumed per mole of vanadate in the TR-promoted oxidation of NADPH to $NADP^+$. When there was less vanadate ion than NADPH, the total change in A_{340} was proportional to the initial [NADPH]. Thus, the reaction is not a 1:1 stoichiometric reaction of vanadate ion with NADPH (in contrast to the case for trypanothione or glutathione disulfides as substrates with their respective cognate enzymes). The vanadate ion appears to have a catalytic role in NADPH turnover. This observation led us to consider whether vanadium un-

dergoes TR-catalyzed cyclical reduction–oxidation with concomitant production of superoxide ion ("redox cycling") as observed for some quinones and nitrofurans with TR.[6–8] Superoxide ion production was monitored by coupling radical formation to reduction of cytochrome *c* (10 μM) and measuring absorbance changes ($\varepsilon_{550} = 21{,}000\,M^{-1}\,cm^{-1}$).[6] Cytochrome *c* experiments were carried out in 0.1 mM NADPH, 0.5 mM vanadate, and 10 μM cytochrome *c*; after 5 min incubation TR (5 μg/mL) was added and reaction monitored at 550 nm. The cytochrome *c* reduction assay was performed in the presence and absence of superoxide dismutase (10, 1 μg/mL, native and boiled) catalase (10 or 1 μg/mL, native and boiled), DMSO (50 μL), MeOH (50 μL), and specific inhibitors of TR e.g., chlorpromazine and fluphenazine.

When cytochrome *c* (10 μM) was incubated with NADPH (0.1 mM) and vanadate ion (0.5 mM), in pH 7.25 20 mM Hepes buffer there was no detectable reduction of the cytochrome *c*. On addition of TR (5 μg/mL), after a lag of 5 min reduction of cytochrome *c* was observed by the increase in absorbance at 550 nm (ε_M $1.07 \times 10^{-6}\,M{\cdot}min^{-1}$). This indicates the generation of some types of reactive oxygen species. Competitive inhibitors of TR inhibited the cytochrome *c* assay (20 μM chlorpromazine inhibited by $40.7 \pm 1.0\%$ and 20 μM fluphenazine by $17.8 \pm 1.0\%$), indicating that the TR active site, or part of it, is involved in production of reactive oxygen species. There was no detected inhibition of this reaction by dimethyl sulfoxide (0.71 mM), methanol (1.24 mM), catalase ($10\,\mu g{\cdot}mL^{-1}$) or superoxide dismutase ($10\,\mu g{\cdot}mL^{-1}$). In contrast, the NADPH oxidation catalyzed by vanadate ion and TR is inhibited by SOD or catalase. The apparent ineffectiveness of the superoxide scavengers including SOD on the cytochrome *c* reduction might be due to production of additional free radical types in the course of the TR-stimulated reaction, (e.g. $RS^{\bullet}$, $HO^{\bullet}$) that can be formed by several mechanisms.[13] Not all of these can be blocked by SOD or other scavengers.

The most likely explanation of the apparent substrate behavior of vanadate ion with TR and NADPH is not that vanadate serves as a disulfide substrate alternative. It is likely that the active site dithiol generates superoxide ion and then other reactive radical species in a Fenton-like reaction. This destroys the NADPH supply and produces reactive species, including reactive oxygen species. As nifurtimox and other anti-trypanosomal drugs are likely to act through their production of superoxide ion, vanadium species might be useful to augment the effects of such drugs against *T. cruzi*. Thus, we synthesized oxovanadium complexes (*bis*-peroxovanadium-1,10-phenanthroline,[19] *bis*-peroxovanadium-bipyridine,[20] *bis*-peroxovanadium-oxalate,[21] and *bis*(maltolato)oxovanadium (IV)[22,23]), but they were not TR inhibitors even at 6 mM.

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References

1 A. H. Fairlamb, P. Blackburn, P. Ulrich, B. T. Chait, and A. Cerami, *Science*, **227**, 1485 (1985).
2 T. J. Benson, J. H. McKie, J. Garforth, A. Borges, A. H. Fairlamb, and K. T. Douglas, *Biochem. J.*, **286**, 9 (1992).
3 R. L. Krauth-Siegel and G. H. Coombs, *Parasitol Today*, **15**, 404 (1999).
4 S. E. Austin, M. O. Khan, and K. T. Douglas, *Drug Des. Discovery*, **16**, 5 (1999).
5 R. L. Krauth-Siegel, H. Bauer, and R. H. Schirmer, *Angew. Chem., Int. Ed.*, **44**, 690 (2005).
6 G. B. Henderson, P. Ulrich, A. H. Fairlamb, I. Rosenberg, M. Pereira, M. Sela, and A. Cerami, *Proc. Natl. Acad. Sci. U.S.A.*, **85**, 5374 (1988).
7 N. K. Cenas, D. Arscott, C. J. Williams, Jr., and J. S. Blanchard, *Biochemistry*, **33**, 2509 (1994).
8 N. Cenas, D. Bironaite, E. Dickancaite, Z. Anusevicius, J. Sarlauskas, and J. S. Blanchard, *Biochem. Biophys. Res. Commun.*, **204**, 224 (1994).
9 P. J. Stankiewicz, A. Stern, and A. J. Davison, *Arch. Biochem. Biophys.*, **287**, 8 (1991).
10 S. Liochev and I. Fridovich, *Arch. Biochem. Biophys.*, **250**, 139 (1986).
11 S. I. Liochev and E. A. Ivancheva, *Free Radical Biol. Med.*, **5**, 349 (1988).
12 S. I. Liochev and I. Fridovich, *Free Radical Biol. Med.*, **6**, 617 (1989).
13 S. I. Liochev and I. Fridovich, *Arch. Biochem. Biophys.*, **279**, 1 (1990).
14 S. I. Liochev and I. Fridovich, *Arch. Biochem. Biophys.*, **294**, 403 (1992).
15 X. Shi and N. S. Dalal, *Arch. Biochem. Biophys.*, **278**, 288 (1990).
16 X. Shi and N. S. Dalal, *FEBS Lett.*, **276**, 189 (1990).
17 X. Shi and N. S. Dalal, *Arch. Biochem. Biophys.*, **302**, 300 (1993).
18 C. Chan, H. Yin, J. Garforth, J. H. McKie, R. Jaouhari, P. Speers, K. T. Douglas, P. J. Rock, V. Yardley, S. L. Croft, and A. H. Fairlamb, *J. Med. Chem.*, **41**, 148 (1998).
19 A. P. Bevan, J. W. Burgess, J. F. Yale, P. G. Drake, D. Lachance, G. Baquiran, A. Shaver, and B. I. Posner, *Am. J. Physiol.*, **268**, E60 (1995).
20 B. I. Posner, R. Faure, J. W. Burgess, A. P. Bevan, D. Lachance, G. Zhang-Sun, I. G. Fantus, J. B. Ng, and D. A. Hall, *J. Biol. Chem.*, **269**, 4596 (1994).
21 B. Barbeau, R. Bernier, N. Dumais, G. Briand, M. Olivier, R. Faure, B. I. Posner, and M. Tremblay, *J. Biol. Chem.*, **272**, 12968 (1997).
22 K. G. Peters, M. G. Davis, B. W. Howard, M. Pokross, V. Rastogi, C. Diven, K. D. Greis, E. Eby-Wilkens, M. Maier, A. Evdokimov, S. Soper, and F. Genbauffe, *J. Inorg. Biochem.*, **96**, 321 (2003).
23 J. H. McNeill, V. G. Yuen, H. R. Hoveyda, and C. Orvig, *J. Med. Chem.*, **35**, 1489 (1992).

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