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Effects of iron depletion on *Entamoeba histolytica* alcohol dehydrogenase 2 (EhADH2) and trophozoite growth: implications for antiamoebic therapy

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***Objectives*: The purpose of this study was to determine the mechanism by which iron chelation affects the trophozoite survival of *Entamoeba histolytica*. Fe^{2+} is a cofactor for *E. histolytica* alcohol dehydrogenase 2 (EhADH2), an essential bifunctional enzyme [alcohol dehydrogenase (ADH) and aldehyde dehydrogenase (ALDH)] in the glycolytic pathway of *E. histolytica*.**

***Methods*: We tested the effects of iron depletion on trophozoite growth, the kinetics of iron binding to EhADH2, and the activities of ADH and ALDH.**

***Results*: Growth of *E. histolytica* trophozoites, and ADH and ALDH enzymatic activities were directly inhibited by iron chelation. Kinetics of iron binding to EhADH2 reveals the differential iron affinity of ADH (higher) and ALDH (lower).**

***Conclusions*: This study demonstrates that iron chelation interrupts the completion of the fermentative pathway of *E. histolytica* by removing the metal cofactor indispensable for the structural and functional stability of EhADH2, thus affecting trophozoite survival. We propose that iron-starvation-based strategies could be used to treat amoebiasis.**

Keywords: AdhE, enzyme inhibition, *E. histolytica*

Introduction

Entamoeba histolytica infects 50 million people yearly causing 100 000 fatalities worldwide.[1] *E. histolytica*'s cysts are ingested in contaminated food or water; trophozoites colonize the host's large intestine causing disentery.[2] Amoebiasis is treated with metronidazole; however, its usefulness is limited by high toxicity,[2] which prompts the search for alternative drugs.

E. histolytica lacks mitochondria and obtains energy by fermenting glucose. The last stages of this pathway convert acetyl-CoA into ethanol by the enzymatic activities of aldehyde dehydrogenase (ALDH) and alcohol dehydrogenase (ADH).[3] These functions are fused in *E. histolytica* alcohol dehydrogenase 2 (EhADH2), which is essential for the survival of *E. histolytica*.[3] AdhE, a homologous *Escherichia coli* enzyme, is required for bacterial fermentative growth. Episomal expression of *ehadh2* gene in *E. coli* complements an *adhE* knock-out mutation, providing a system for inhibitor identification.[3,4] Site-directed mutagenesis of conserved histidines at the iron-binding domain of EhADH2 inactivates ADH and ALDH, thus rendering the mutant proteins unable to rescue anaerobic growth of *E. coli* SHH31(Δ*adhE*).[3] Although our previous analyses suggest that iron is important for amoebic growth and enzymatic activities,[3] the physiological role of iron in amoebic growth or EhADH2 function had not been elucidated. Studies have emphasized the importance of iron in pathogenic diseases and suggested iron chelation as chemotherapy, based on mammalian immune responses that sequester iron as the first line of defense.[5] This study shows that iron affects ADH and ALDH enzymatic activities and trophozoite survival. We propose that iron starvation could be further explored as treatment for amoebiasis.

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Materials and methods

E. histolytica strain/growth conditions

E. histolytica strain HM1:1MSS trophozoites were cultured under axenic conditions as described previously.[3] Initial inoculations of 5×10^3 trophozoites/tube were grown for 48, 72 and 96 h and counted using a haemocytometer.

Metal/chelation effects on E. histolytica's trophozoite growth

Amoebic trophozoites (5×10^3) were grown for 48, 72 and 96 h in TYI-S-33 alone or with 30 μM ferrous sulphate, 50 μM zinc sulphate, 30 μM 1,10-phenanthroline or a combination of 30 μM ferrous sulphate + 30 μM 1,10-phenanthroline. To measure the reversal of the chelators' inhibitory effect on growth of *E. histolytica*, amoebic trophozoites were grown with 30 μM 1,10-phenanthroline for 7 h, then exposed to 30 μM ferrous sulphate, 30 μM ferrous sulphate + 30 μM zinc sulphate + 30 μM 1,10-phenanthroline or 30 μM 1,10-phenanthroline, or left in TYI medium alone (control) for 7, 24 and 40 h, and counted.

Assessment of EhADH2 ADH and ALDH activities

Bacterial cells were processed as described elsewhere.[3,4] Bacterial lysates were used to measure NAD^+-dependent ALDH and ADH activities. ALDH activity was assayed spectrophotometrically by measuring the decrease in absorbance at 340 nm, following the oxidation of NADH to NAD using acetyl-CoA as a substrate.[3,4] ADH activity was measured similarly, by substituting acetaldehyde for acetyl-CoA as a substrate.[3,4] One unit of enzyme activity is defined as that which consumed 1 mmol of NADH or NAD^+/min. Activity values were averaged from three independent experiments. All samples were confirmed by SDS–PAGE and western blot analyses.[3]

Effect of metal cofactors on EhADH2 catalytic activity

Fe^{2+} content of bacterial lysates was determined colorimetrically using 1,10-phenanthroline as an indicator with an extinction coefficient of 31.5 mM/cm at 510 nm.[6] EhADH2 was added to an excess of 1.5 mM (3-fold) 1,10-phenanthroline in 100 mM Trizma, pH 7.5. The 1,10-phenanthroline Fe^{2+} complexes were removed by dialysis

Table 1. EhADH2 enzymatic activities are rescued by adding iron

Metal/chelator additives	ADH (U/mg)	ALDH (U/mg)
M9 medium only	1.31 ± 0.52	0.41 ± 0.19
30 μM Fe^{2+}	27.88 ± 2.40	7.67 ± 1.19
50 μM Zn^{2+}	0.23 ± 0.17	−0.03 ± 0.3
30 μM 1,10-phenanthroline	0.71 ± 0.46	−0.15 ± 0.8
30 μM 1,10-phenanthroline + 30 μM Fe^{2+}	28.83 ± 1.14	9.21 ± 1.24
30 μM 1,10-phenanthroline + 30 μM Fe^{2+} + 50 μM Zn^{2+}	22.67 ± 2.51	8.56 ± 0.39

NAD-dependent ADH and ALDH activities were measured from partially purified bacterial lysates of *E. coli* SHH31 expressing wild-type recombinant EhADH2 enzyme to which indicated metals, chelator or combined additives were added.

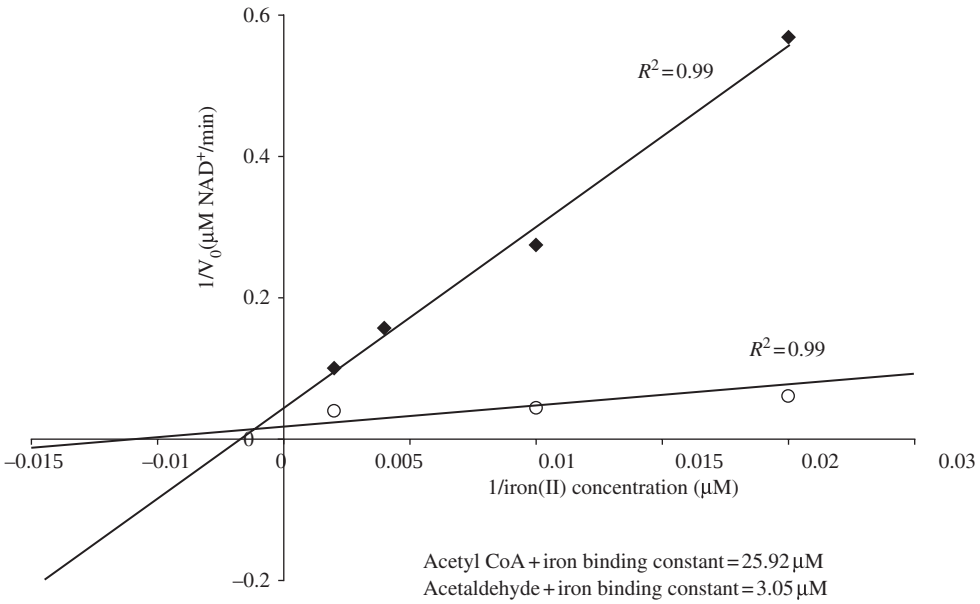

Figure 1. Iron-binding constants. Double reciprocal plot displaying initial rate of NADH-dependent acetyl-CoA and acetaldehyde dehydrogenase reaction as a function of Fe^{2+} concentration. Fe^{2+} was removed from partially purified EhADH2 with phenanthroline. The concentration of Fe^{2+} present in the standard activity assay buffer was then varied between 10 and 500 μM, and the initial rate of reaction was measured. Lineweaver–Burk plots were constructed and constants were calculated. Acetyl-CoA + Fe^{2+}, filled diamonds; acetaldehyde + Fe^{2+}, open circles.

against 20 mM Tris–HCl, pH 7.5. ADH and ALDH activities were measured with no ferrous sulphate, 30 μM ferrous sulphate, 50 μM zinc sulphate, 30 μM 1,10-phenanthroline or a combination of 30 μM ferrous sulphate + 30 μM 1,10-phenanthroline. All assays were repeated in triplicate.

Fe^{2+} affinity and maximum rate

As iron is not strictly a substrate, we use the term 'binding affinity constant' instead of K_m. It is inferred that a lower binding affinity constant suggests stronger affinity for iron. The apparent Michaelis–Menten constant for iron binding and the maximum rate of reaction were determined as follows: Fe^{2+} was removed from partially purified EhADH2.[6] The concentration of Fe^{2+} present in the standard activity assay buffer was varied between 10 and 500 μM, and the initial rate of reaction was measured. Constant values were calculated.

Results

Iron effects on EhADH2 expression and enzymatic activities

ADH and ALDH activities were enhanced by iron and inhibited by zinc or phenanthroline (Table 1). Iron in combination with phenanthroline or zinc 'rescued' enzymatic activities to levels similar to ones with iron alone (Table 1). SDS–PAGE showed that the effect of iron was not due to altered EhADH2 expression levels (data not shown). The affinity of EhADH2 for iron + acetaldehyde (lower binding constant = 3.05 μM) was 8.5 times stronger than for iron + acetyl-CoA (25.9 μM) (Figure 1). V_{max} values were calculated as the maximum rate obtainable by Fe^{2+} saturation in the presence of 0.2 mM acetyl-CoA or 0.5 mM acetaldehyde and 0.2 mM NADH (data not shown).

Physiological effect of iron on E. histolytica trophozoite growth

As shown in Figure 2(a), zinc and phenanthroline reduced trophozoite growth 1.5-fold compared with medium alone, whereas iron enhanced trophozoite growth 2.5-fold. Iron counteracted the inhibitory effects of phenanthroline and zinc when added together (Figure 2a). Iron starvation by phenanthroline was reversed by iron even after several hours of growth in the presence of chelator (Figure 2b). Initially, the effects of iron starvation on *E. coli*$_{pEhADH2}$ growth were tested. Iron chelation inhibited the EhADH2-expressing *E. coli*; supplemental iron was able to reverse phenanthroline/zinc inhibition (data not shown).

Discussion

Our study shows that EhADH2 requires Fe^{2+} and is inhibited by phenanthroline/zinc (Table 1 and Figure 1). Zinc, although a divalent cation, is unable to substitute for Fe^{2+}. EhADH2 activities can be 'rescued' in the presence of chelator by adding saturating Fe^{2+}. The increase in catalytic activity in the Fe^{2+}–chelator combination (Table 1) can be explained by this saturation. Fe^{2+} forms a complex with phenanthroline in a ratio of 1:3. When 30 μM Fe^{2+} and 30 μM phenanthroline were added, 20 μM Fe^{2+} remained in solution, increasing both activities. The apparent Michaelis–Menten constant for iron binding in the presence of

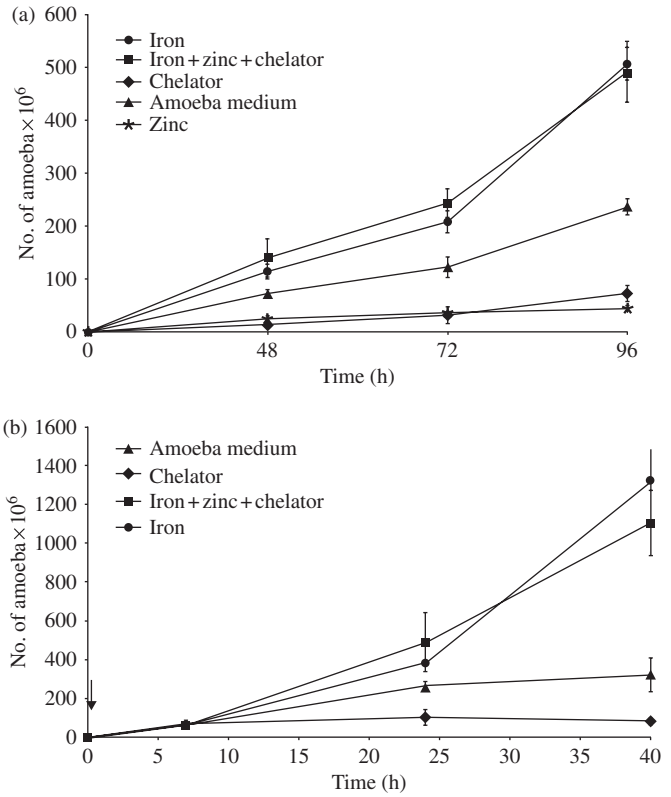

Figure 2. (a) Inhibition of trophozoite growth by iron starvation. An initial 5×10^3 HM1:1MSS *E. histolytica* trophozoites were inoculated per tube with TYI medium alone (filled triangles), 30 μM ferrous sulphate (filled circles), 1,10-phenanthroline (filled diamonds), 50 μM zinc sulphate (asterisks) or 30 μM ferrous sulphate + 50 μM zinc sulphate + 1,10-phenanthroline (filled squares), and counted at 48, 72 and 96 h. (b) Chelator-dependent inhibition is reversed by iron saturation. An initial 5×10^3 HM1:1MSS *E. histolytica* trophozoites were inoculated per tube of TYI medium with 30 μM 1,10-phenanthroline for 7 h. After this initial exposure to the chelator, trophozoites were exposed to 30 μM ferrous sulphate (filled circles), 30 μM 1,10-phenanthroline (filled diamonds) or 30 μM ferrous sulphate + 50 μM zinc sulphate + 30 μM 1,10-phenanthroline (filled squares), or left in TYI medium alone (filled triangles), and counted at 7, 24 and 40 h. The time when second additions were supplemented (after the 7 h) is indicated by an arrow.

acetaldehyde and acetyl-CoA is seen in Figure 1. A lower apparent Michaelis–Menten constant for iron (3.1 μM) with acetaldehyde confirms the importance of Fe^{2+} in *E. histolytica*'s conversion of acetaldehyde into ethanol. However, we also detected an apparent Michaelis–Menten constant for iron in the presence of acetyl-CoA (26 μM), which suggests that the ALDH activity requires an intact N-terminus and C-terminus (Table 1, Figure 1 and site-directed mutagenesis[3]). A recent study showed that *E. coli* SHH31, transformed with the N-terminal ALDH domain, retains 30% of ALDH enzymatic activity compared with the wild-type EhADH2.[7] Based on our findings, we conclude that EhADH2 could have diverged structurally/functionally from the ancestral enzyme, as ADH and ALDH depend on iron binding for activity. Alternatively, an additional iron-binding site (and more than one iron) could be involved in the reaction as the K_m for NADH is similar for acetaldehyde and acetyl-CoA.[3]

Iron increased *E. histolytica* trophozoite growth (Figure 2a). Growth inhibition of amoebic trophozoites by phenanthroline/zinc was countered by supplementary iron (Figure 2a). Although

iron-containing superoxide dismutase (Fe^{2+}SOD) is probably the main enzyme for detoxification of reactive oxygen intermediates,[2] scavenging iron might change EhADH2 conformation and delay/prevent oxygen inactivation (Figure 2b). We delayed ADH/ALDH oxygen damage by lysing the cells with phenanthroline, thus restoring activity by adding iron prior to the assay (data not shown). *E. coli*'s AdhE is inactivated by hydrogen peroxide due to a metal-catalysed oxidation reaction.[8] AdhE seems to act as an H_2O_2 scavenger in *E. coli* cells delaying death.[8] Inactivation of iron transcriptional regulator RitR in *Streptococcus pneumoniae* decreases expression levels of *adhE* mRNA and increases susceptibility to hydrogen peroxide during exponential growth.[9] The role of AdhE homologues might be to prevent DNA damage and protect pathogens from oxidative stress.

Our work implies that sequestering iron for the activities of EhADH2 (Table 1 and Figure 1) could be used to control amoebic infection. Previous studies have shown associations between amoebic infection and iron levels in humans: milk-drinking Maasai have less amoebic infections than consumers of a mixed diet (milk and blood), suggesting that low-iron-content foods could provide some resistance to amoebiasis.[10] Higher incidence of amoebic liver abscess in alcoholic patients has been correlated with increased hepatic iron content.[11] Interestingly, immune responses to pathogens in mammals rely on iron sequestration.[5]

Recent studies have linked the iron scavenging role of lactoferrin/lactoferricin to trophozoite death *in vitro*,[12] implying that controlled iron depletion could be used to kill amoeba. Researchers have speculated that the antiamoebic effectiveness of iodo-hydroxyquinolines relies on its iron-binding properties.[13] Our results demonstrate that iron chelation interrupts the completion of the fermentative pathway of *E. histolytica* by removing the metal cofactor indispensable for the structural and functional stability of EhADH2. *In vivo* mammal experimentation shall be pursued to establish the appropriate use of iron chelation against amoebiasis.

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Transparency declarations

None to declare.

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