Recombinant expression and biochemical characterization of an NADPH:flavin oxidoreductase from *Entamoeba histolytica*

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The gene encoding a putative NADPH:flavin oxidoreductase of the protozoan parasite *Entamoeba histolytica* (Eh34) was recombinantly expressed in *Escherichia coli*. The purified recombinant protein (recEh34) has a molecular mass of about 35 kDa upon SDS/PAGE analysis, exhibits a flavoprotein-like absorption spectrum and contains 1 mol of non-covalently bound FMN per mol of protein. RecEh34 reveals two different enzymic activities. It catalyses the NADPH-dependent reduction of oxygen to hydrogen peroxide (H_2O_2), as well as of disulphides such as 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB) and cystine. The disulphide reductase but not the H_2O_2-forming NADPH oxidase activity is inhibitable by sulphydryl-active compounds, indicating that a thiol component is part of the active site for the disulphide reductase activity, whereas for the H_2O_2-forming NADPH oxidase activity only the flavin is required. Compared with the recombinant protein, similar activities are present in amoebic extracts. Native Eh34 is active in a monomeric as well as in a dimeric state. In contrast to recEh34, no flavin was associated with the native protein. However, both NADPH oxidase as well as DTNB reductase activity were found to be dependent on the addition of FAD or FMN.

INTRODUCTION

The protozoan *Entamoeba histolytica*, the causative agent of human amoebiasis, normally resides and multiplies within the human gut under anaerobic or microaerophilic conditions. However, *E. histolytica* is able to invade the intestinal mucosa and disseminates to other organs, most commonly to the liver, and produces abscesses [1]. During invasion, *E. histolytica* is exposed to an increased oxygen pressure.

E. histolytica lacks a functional tricarboxylic acid cycle, cytochromes and does not have a conventional respiratory electron transport chain terminating in the reduction of oxygen to water. However, *E. histolytica* does respire and earlier studies indicated that the parasite can tolerate up to 5% oxygen in the gas phase [2,3].

In 1980, Lo and Reeves [4] reported on the purification of an NADPH:flavin oxidoreductase from *E. histolytica* lysates. Under aerobic conditions the purified enzyme passes the reducing equivalents from reduced flavin to oxygen to form hydrogen peroxide (H_2O_2). However, amoebae do not produce detectable amounts of H_2O_2. Therefore, the authors speculated that the enzyme produces water *in vivo* via an electron carrier intervening between oxygen and reduced flavin. Possibly oxygen is toxic for the parasite and, therefore, NADPH:flavin oxidoreductase serves as a scavenger to reduce the amount of oxygen to an acceptable level [4].

We recently reported on the isolation of an *E. histolytica* gene encoding a protein with a calculated molecular mass of 34 kDa (Eh34; *E. histolytica* NADPH:flavin oxidoreductase), which has substantial similarity to a class of disulphide oxidoreductases, so far reported for prokaryotic species only [5]. Comparison of the deduced amino acid sequence revealed identities of about 30–40% to the thioredoxin reductases of *Escherichia coli*, *Streptomyces clavuligens* and *Penicillium chrysogenum*, to the AhpF subunit of the alkyl hydroperoxide reductase system (AhpR) of *S. typhimurium* and *E. coli*, as well as to the H_2O_2-forming NADH oxidase of *Amphibacillus xylanus* [6–10]. Although, all of the various proteins are flavoproteins that catalyse the reduction of DTNB in an NADH- or NADPH-dependent manner, they all have different functions within the cell. The thioredoxin system, which consists of thioredoxin reductase and thioredoxin, is involved in several biological pathways. It acts as a hydrogen donor for various enzymes, it removes reactive oxygen species and regulates enzymic activities by controlling the thiol-redox state within the cell [11,12]. AhpF together with AhpC constitute an antioxidant system, which reduces and therefore detoxifies H_2O_2 and organic hydroperoxides [13–15]. The NADH oxidase from *A. xylanus* seems to be important for the generation of NAD^+ from NADH, which is produced during glycolysis and pyruvate oxidation under aerobiosis of this facultative anaerobic bacterial species [10,16].

In the present paper, we report on recombinant expression and characterization of Eh34. Our studies indicate that the amoebic protein functions as a disulphide reductase as well as a H_2O_2-forming NADPH oxidase.

MATERIALS AND METHODS

Recombinant expression and purification of Eh34

Two synthetic oligonucleotide primers, Eh34-S28 (5′-CCA AAA AAA TCA TAT GAG TAA TAT TCA T) and Eh34-AS30 (5′-GTT AAA AAG GAT CCT GAA TTA ATG AGT TTG), were used for PCR amplification of the gene encoding Eh34. The primers contain either *Nde*I or *BamH*I restriction sites, which were used for rapid cloning of the amplified DNA in a predicted orientation into the prokaryotic expression plasmid pJC45. pJC45 is a derivative of pJC40 and allows expression of recombinant proteins with the addition of 10 N-terminal histidine residues [17]. Recombinant plasmids were transformed into the *E. coli* strain Bl21(DE3)[pAPlacIQ] and subsequently bacteria were plated on Luria broth agar plates [100 μg/ml ampicillin, 50 mg/μl kanamycin, 2% (w/v) glucose]. Freshly transformed

Abbreviations used: Eh34, *Entamoeba histolytica* NADPH:flavin oxidoreductase; recEh34, recombinantly expressed Eh34; Ahp, alkyl hydroperoxide reductase; ABTS, 2,2′-azinobis-(3-ethylbenzthiazolinesulphonic acid); H_2O_2, hydrogen peroxide; DTNB, 5,5′-dithiobis-(2-nitrobenzoic acid).

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single colonies were inoculated into Luria broth medium and grown at 37 °C until OD_{600} reached 0.3. Subsequently isopropyl-β-D-thiogalactoside was added (final concentration 1 mM) and incubation was continued for an additional 3 h. Purification of the recombinant protein was achieved using an Ni-NTA-resin (Qiagen GmbH, Hilden, Germany) according to the purification manual for soluble proteins described by the pET system (Novagen). Briefly, the bacterial pellet was resuspended in binding buffer (5 mM imidazole, 0.5 M NaCl, 20 mM Tris/HCl, pH 7.9) and sonified. The lysate was centrifuged (40000 ***g***, 30 min) to remove cell debris. The Ni-NTA agarose column was loaded with the prepared extract, washed with 10 volumes binding buffer and 10 vol. of wash buffer (60 mM imidazole, 0.5 M NaCl, 20 mM Tris/HCl, pH 7.9). The recombinant protein was eluted with elution buffer (1 M imidazole, 0.5 M NaCl, 20 mM Tris/HCl, pH 7.9). For further experiments the recombinant protein was dialysed against the corresponding buffer.

Enzyme assays

NAD(P)H : flavin oxidoreductase

NAD(P)H : flavin oxidoreductase activity was determined as described by Lo and Reeves [4]. To study the activity of the recombinant protein the addition of FMN or FAD is not necessary. The activity was assayed by measuring the initial rate of NAD(P)H oxidation at 340 nm ($\epsilon = 6.22$ M/cm) at 25 °C. One unit of NAD(P)H : flavin oxidoreductase activity was defined as the amount of enzyme which catalyses the oxidation of 1 μmol NAD(P)H/min.

Disulphide reductase

Disulphide reductase activity was determined using the DTNB (5,5′-dithiobis-(2-nitrobenzoic acid); Fluka) assay as described by Holmgren [18]. The assay mixture contained 0.1 M potassium phosphate (pH 7.0), 2 mM EDTA, 0.05–0.2 mM NADPH or NADH and 10 mM DTNB. DTNB had been dissolved in DMSO (1 M stock solution). To study the activity of the native enzyme the addition of 0.025 mM FMN or FAD is necessary. Enzyme was added to initiate the reaction and the change in absorbance at 412 nm was monitored. The activity was calculated as μmol NADPH oxidized per min according to $A_{412}/(27.2)$ since 1 mol of NADPH yields 2 mol of TNB ($\epsilon_{412\text{nm}}$ DTNB = 13.6 M/cm). The cystine reductase activity was calculated as μmol NADPH oxidized per min at 340 nm. A final concentration of 5 mM cystine was used. All reactions were done at least in duplicate.

Determination of H_2O_2 formation

Two spectrophotomethric methods were used to determine H_2O_2 formation at various time points (1 to 30 min) during the NADPH : flavin oxidoreductase reaction. Ferrithiocyanate method [19]: the reactions were terminated by addition of trichloroacetic acid and centrifuged at 12000 ***g***. Subsequently, 0.2 vol. of 10 mM ferrous ammonium sulphate and 0.1 volume of 2.5 M potassium thiocyanate were added. In the presence of H_2O_2, Fe^{2+} becomes oxidized, resulting in a coloured thiocyanate–Fe^{3+} complex which was measured by its absorption at 480 nm.

ABTS [2,2′-azinobis-(3-ethylbenzthiazolinesulphonic acid)] method [20]: after the oxidoreductase reaction had proceeded for different time points the H_2O_2 formation was determined by the addition of 20 mM ABTS (5 μl) and 20 U horseradish peroxidase to a final volume of 500 μl. The amount of H_2O_2 was determined spectrophotometrically (414 nm; $\epsilon = 36 \times 10^3$ M/cm) taking known amounts of H_2O_2 (10–300 nmol) as standard.

The effects of sulphydryl-dependent inhibitors were determined by pre-incubation of recEh34 (recombinantly expressed Eh34) with 0.1–5 mM arsenite or iodoacetamide for 10 min prior to addition to the various assays. Control experiments were performed using arsenite and iodoacetamide without adding the enzyme.

Enzymic staining of native polyacrylamide gels

After electrophoresis of purified recEh34 or amoebic extract using an 8 % non-denaturing polyacrylamide gel, the gel was incubated in 10 mM Tris/HCl buffer, pH 8.0, for 10 min and for an additional 5 min in the same buffer supplemented with 0.25 mM NADPH or NADH. Subsequently, the gel was incubated in 10 mM Tris/HCl buffer, pH 8.0, 0.25 mM NADPH or NADH, 5 mg/ml Nitro Blue Tetrazolium until NAD(P)H oxidase bands became visible.

Prosthetic group identification

Flavin was liberated from the recombinant enzyme by boiling for 10 min and separated from the protein by centrifugation at 14000 ***g*** for 10 min. To determine whether FAD or FMN forms the prosthetic group, excitation and emission wavelengths at 450 nm and 535 nm were measured at pH 2.6 and pH 7.9, according to Faeder and Siegel [21], using a fluorescence spectrophotometer (model MPF-2A; Perkin–Elmer LS50).

Molecular mass determination of native Eh34

The molecular mass of Eh34 was determined using gel filtration. The *E. histolytica* isolate HM-1 : IMSS was cultured in TYI-S-33 medium in the absence of bacteria (axenically) [22]. About 3×10^8 cells at the late-exponential phase of growth were harvested by chilling on ice for 10 min and centrifuged at 430 ***g*** at 4 °C for 5 min. The resulting pellet was washed twice in PBS, freeze–thawed five times in solid CO_2/ethanol and sedimented by centrifugation at 150000 ***g*** at 4 °C for 40 min. The 150000 ***g*** supernatant was passed over a Hi/Load Superdex 200 HR 16/60 FPLC column (Pharmacia, Uppsala, Sweden) with 50 mM Tris/HCl buffer, pH 8.0. The fractions were tested for activity and stored at 4 °C. Active fractions were tested in Western blot analyses using a rabbit antiserum raised against recEh34. SDS/PAGE in 12 % gels and subsequent immunoblots were carried out as described [23].

Rabbit antiserum

Antiserum to recEh34 was obtained by subcutaneous immunization of a rabbit with 50 μg of purified recEh34 emulsified in complete Freund's adjuvant followed by three booster immunizations at intervals of 14 days with the same amount of protein emulsified in incomplete Freund's adjuvant.

RESULTS

Recombinant expression of Eh34

The gene encoding Eh34 was ligated into a prokaryotic expression vector, allowing recombinant expression as an N-terminally histidine-tailed fusion protein in *E. coli*. Recombinant Eh34 was purified from *E. coli* lysates by metal chelate chromatography using an Ni-NTA resin, yielding about 8 mg of purified recEh34 per litre of bacterial culture. Identity of the recombinant protein was confirmed by N-terminal sequencing. Upon SDS/PAGE analysis, under reducing conditions, recEh34 migrated as a single protein with a molecular mass of 35 kDa, which is in agreement

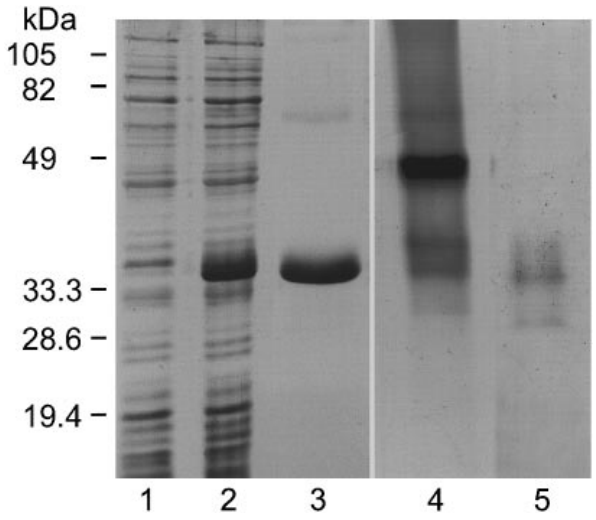

Figure 1 Expression and purification of recombinant Eh34

Expression and purification is monitored by a 12% SDS/PAGE under reducing conditions with 25 mM DTT (lanes 1 to 3 and 5) or under non-reducing conditions (lane 4) and silver stained. Lane 1, lysate of *E. coli* transformed with pJC45 (40 μg, control); lane 2, lysate of *E. coli* transformed with pJC45 containing the Eh34 cDNA (40 μg); lane 3, recEh34 after purification by Ni-NTA agarose (0.8 μg); lane 4, purified recEh34 under non-reducing conditions (5 min at room temperature, 0.8 μg); lane 5, eluted and subsequently reduced 50 kDa protein of purified recEh34 as obtained under non-reducing conditions (see lane 4).

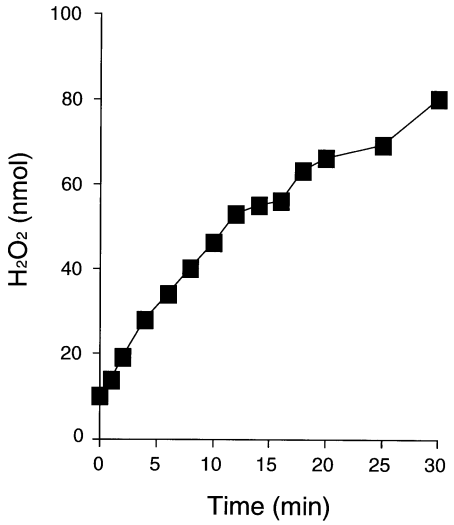

Figure 2 H_2O_2 formation of recEh34

Using the ferrithiocyanate method, the amount of H_2O_2 produced by 0.4 μg of recEh34 was determined after various periods of incubation. The results are expressed as averages of duplicate assays.

with the calculated molecular mass of Eh34 as deduced from the DNA-derived amino acid sequence plus 10 histidine residues that form the N-terminal histidine tail. Under non-reducing conditions, purified recEh34 revealed two bands, of 35 kDa and 50 kDa. After electroelution from the gel and subsequent reduction, the 50 kDa protein disappeared and only a single protein of 35 kDa was detected, indicating that the 50 kDa protein constitutes a homodimer of recEh34 (Figure 1).

Physical properties of recEh34

The recombinant enzyme exhibits an absorption spectrum typical for flavoproteins. Two major peaks at 370 and 450 nm were observed with a shoulder at 470 nm. Under anaerobic conditions, the peak at 450 nm disappeared after addition of 0.5 mM NADPH, which is in line with spectra typical for flavoproteins and NAD(P)H oxidases. Denaturing of recEh34 by boiling resulted in the release of the flavin, indicating a non-covalent association with the protein. The fluorescence intensity of free flavin fraction at pH 2.6 revealed a 1.5-fold decrease in comparison with the control at pH 7.9. This indicates that FMN rather than FAD forms the prosthetic group in recEh34. For recEh34 it was calculated that 28.68 nmol FMN (ϵ_{450nm} FMN = 12.2×10^3 M/cm, A_{450} of purified recEh34 = 0.35) is associated with 29.94 nmol of recEh34 (protein concentration of purified recEh34 = 1.1 mg/ml, calculated molecular mass of recEh34 = 36.74 kDa). Therefore, 0.96 mol FMN is bound per mol of recEh34.

Enzymic properties of recEh34

Oxidation of NAD(P)H

Purified recEh34 revealed NADPH:flavin oxidoreductase activity, with V_{max} of 8 μmol/min per mg of protein and a k_{cat} of 306 min^{-1} at pH 8.0. Highest enzyme activity was obtained at a pH between 7.5 and 9.0, whereas enzymic activity decreased to V_{max} of 1.5 μmol/min per mg of protein at pH values of 6 and 10. Almost no activity was detected using NADH instead of NADPH as substrate (K_m for NADPH = 55 μM). The enzymic activity was stable at 4 °C over several weeks, but was lost after heating at 60 °C for 10 min.

H_2O_2 formation

Under aerobic conditions recEh34 produced H_2O_2 during the NADPH:flavin oxidoreductase reaction (Figure 2). Irrespective of whether the ABTS or the ferrithiocyanate method was used, 1 mg of recEh34 was found to produce 11 μmol of H_2O_2 per min. No H_2O_2 formation was obtained using heat-denatured recombinant protein (10 min/60 °C).

NADPH-dependent disulphide reductase activity

In addition to NADPH:flavin oxidoreductase activity, recEh34 was found to be able to catalyse the reduction of disulphides such as cystine or DTNB. The calculated V_{max} for the DTNB reductase reaction was 10 μmol/min per mg of protein and k_{cat} was 360 min^{-1}. Maximal activity was obtained at 10 mM DTNB with an apparent K_m value for DTNB of 500 μM and for NADPH of 0.5 μM. Reduction of DTNB was absolutely dependent on the presence of NADPH. Likewise, the reduction of cystine was also NADPH-dependent. Without NADPH or in the presence of NADH no activity was observed. In the presence of 5 mM cystine V_{max} was calculated to be 24 μmol/min per mg of protein.

The two reagents, arsenite and iodoacetamide, commonly used to inhibit sulphydryl-dependent reaction were tested for their ability to inhibit enzymic properties of recEh34. Both compounds were found to inhibit the reduction of DTNB (1 mM iodoacetamide leads to 50% inhibition of recEh34 and 83% inhibition of Eh34; 1 mM arsenite leads to 60% inhibition of recEh34 and 65% inhibition of Eh34), whereas NADPH oxidase activity was not altered. This indicates that Eh34 contains a thiol component which is part of the active-site responsible for DTNB reduction, but which is not required for NADPH-oxidase activity.

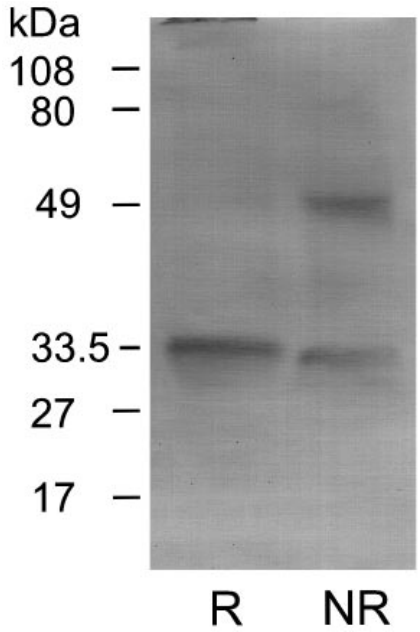

Figure 3 Identification of native Eh34 in amoebic lysates

Extracts of amoebae were separated on a 12% SDS/PAGE under reducing (R) or non-reducing (NR) conditions and analysed by immunoblotting using an antiserum raised against recEh34.

Molecular mass determination and biochemical characterization of native Eh34

An antiserum raised against recEh34 was used to identify native Eh34 in *E. histolytica* lysates. Western blots performed with reduced amoebic extracts revealed a single protein of 34 kDa, whereas under non-reducing conditions two proteins with molecular masses of about 34 and 50 kDa were detected (Figure 3). In addition, the molecular mass of native Eh34 was determined by gel filtration using a Superdex 200 column previously equilibrated with proteins of known molecular mass. Two peaks, both containing NADPH : flavin oxidoreductase as well as H_2O_2-forming activity were obtained, which corresponded to proteins with molecular masses of 40 kDa and 80 kDa. Under reducing conditions, both active fractions contain a protein of 34 kDa, reacting with anti-recEh34 in Western blots. Gel filtration of recEh34 resulted in activity peaks corresponding to proteins of about the same size (35 and 65 kDa) as determined for native Eh34. In contrast to the recombinant protein, native Eh34 required the addition of FMN or FAD to exhibit the highest specific activity. No differences in specific activities were observed using FAD or FMN as cofactor.

In addition, NADPH oxidase activity of recombinant as well as of native Eh34 was identified by their ability to reduce *p*-Nitro Blue Tetrazolium on non-denaturing polyacrylamide gels. Western blot analyses of these gels using anti-recEh34 antiserum revealed labelling of protein bands identical in size with those visualized by activity staining (Figure 4).

Oxidoreductase activity was detected in soluble but not in membrane fractions of *E. histolytica* extracts, which is in line with results obtained by indirect immunofluorescence microscopy, indicating that Eh34 is localized within the cytoplasm of the amoebae (results not shown).

DISCUSSION

We made use of recombinant expression to characterize the *E. histolytica* protein Eh34. Since the recombinant protein as well as partially purified fractions of amoeba lysates revealed similar properties it seems reasonable to assume that the recombinant protein is suitable to study the biochemical characteristics of Eh34. Remarkably, Eh34 exhibits two different enzymic properties. It constitutes an NADPH-dependent disulphide reductase as well as an H_2O_2-forming NADPH oxidase. Both activities could be separately investigated using inhibitors known to suppress sulphydryl-dependent reactions, e.g. arsenite and iodoacetamide. Both compounds do not alter H_2O_2-forming NADPH oxidase activity, whereas the disulphide reductase activity was fully repressed. These data indicate that free thiol groups within Eh34 are involved in disulphide reduction but are not required for H_2O_2-forming NADPH oxidation. Unlike other disulphide

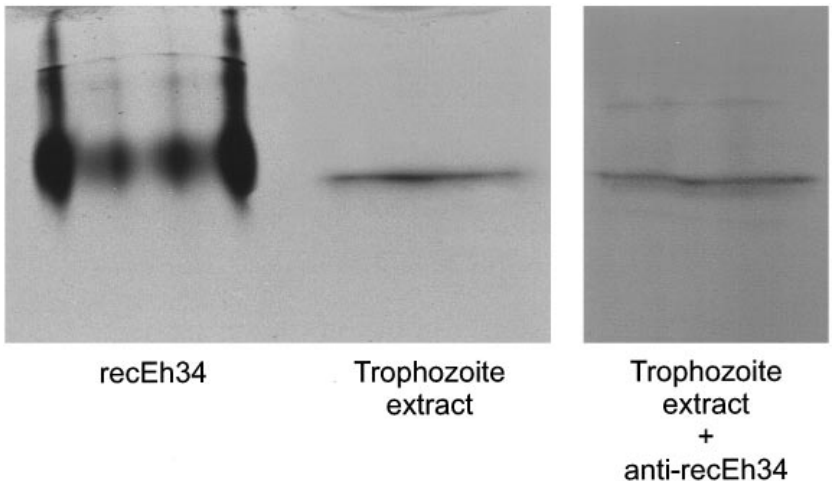

Figure 4 NADPH oxidase activity of recEh34 and native Eh34

The recEh34 or extracts of amoebae were separated on a 12% non-denaturing polyacrylamide gel and stained for NADPH oxidase activity using Nitro Blue Tetrazolium as substrate. In addition, separated amoebic extract was analysed by immunoblotting using anti-recEh34 antiserum.

reductases, which are active as dimers or tetramers only [6,8,19], the amoebic enzyme is active as a monomer as well as a homodimer.

RecEh34 efficiently catalyses the electron transfer from NADPH to different disulphide-containing receptors such as cystine and DTNB. *E. histolytica* trophozoites contain high levels of cysteine and growth of amoebae depends on cysteine being supplemented to the culture medium. However, cysteine can also be replaced by cystine [24]. On the other hand, *E. histolytica*, like other protozoan parasites such as *Giardia lamblia* or *Trichomonas* species, lacks glutathione and glutathione-dependent enzymes [25–28]. Therefore, a disulphide reductase in *E. histolytica* has been postulated for the reduction of cystine to cysteine [29]. It seems reasonable to assume the Eh34 represents the postulated enzyme. Beside disulphide reductase activity, Eh34 has H_2O_2-forming NADPH oxidase activity. Structurally related proteins with similar enzymic properties have been described for *S. typhimurium* (AhpF) and *A. xylanus* (NADH oxidase) [9,13,16,31]. AhpF together with the smaller subunit AhpC constitute an alkyl hydroperoxide reductase (AhpR) system of *S. typhimurium*. AhpR has been identified as an antioxidant system capable to reduce alkyl hydroperoxides and H_2O_2. It is believed that the *A. xylanus* protein is also part of an AhpR system. *A. xylanus* NADH oxidase can substitute *S. typhimurium* AhpF to catalyse the NADH-dependent two-electron reduction of cumene hydroperoxide and H_2O_2 in the presence of *S. typhimurium* AhpC. Furthermore, *A. xylanus* NADH oxidase in the presence of *S. typhimurim* AhpC catalyses the four-electron reduction of oxygen to water [31]. An AhpC-like protein, which was termed Eh29, has also been identified in *E. histolytica* [32–34]. Recently, it was shown that Eh34 converts oxygen into H_2O_2 and reduces Eh29, which subsequently is able to remove and thus detoxify the H_2O_2 produced [35]. Therefore, beside the reduction of cystine and DTNB, Eh34 can transfer reducing equivalents to oxygen to form H_2O_2, as well as to Eh29 to convert proteins from their non-active, oxidized form back into its active, reduced form.

As found for the various disulphide oxidoreductases of other organisms, Eh34 is a flavoprotein, which is active only in the presence of flavins. Interestingly, the recombinant enzyme isolated from bacterial lysates contains 1 mol of non-covalently bound FMN per mol of protein, whereas no flavin was found to be associated with the native enzyme from amoebic extracts. However, enzyme activity of the native protein was dependent on the presence of FMN, FAD or riboflavin [4]. In contrast to recEh34 or other NADPH oxidases known so far, which all contain a non-covalently bound flavin, no evidence for the existence of peptide-bound flavins in *E. histolytica* has been detected [36–39], although flavins have been reported to be present within the amoebae in sufficient quantities (0.15 nmol FMN per mg of protein) [36]. At present, we do not have a convincing explanation as to why no flavin was associated with the native Eh34 and for the lack of specificity for flavins. It is possible that during the preparation of amoebic extracts flavin concentrations were reduced or these cofactors were lost during purification of proteins. Therefore, the addition of flavins at nearly the same amount as is present in the amoebic cell (7.6 ± 0.9 μg/g fresh cells) [36] is necessary to get highest specific activity.

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