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An enzyme found in amitochondriate eukaryotic organisms, NAD(P)H oxidoreductase One simpler homologue of the enzyme found in vertebrates is Giardia lamblia.

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Abstract

The enzyme glQR, found in the amitochondriate eukaryotic protozoan Giardia lamblia, catalyses the two-electron transfer oxidation of NAD(P)H using a quinone as an acceptor. G. lamblia expressed the gene for this protein in Escherichia coli. The NAD(P)H oxidoreductase activity was present in the purified recombinant protein, and it was more effective at donating electrons than NADH. The enzyme's substrates included menadione, naphthoquinone, and a number of synthetic electron acceptors. glQR is quite similar in amino acid sequence to other vertebrate homologues and to a number of putative bacterial proteins.The secondary structural parts of glQR are similarly organised in three-dimensional modelling, despite the fact that it is much smaller than mammalian enzymes. Enzymes in mammals have a high degree of conservation among their amino acid residues that are involved in substrate binding and catalysis. Since glQR shares these characteristics with other members of this protein family as well as similarities in substrate selectivity and inhibitor sensitivity, it may be confidently called a member of this family.

Keywords: menadione oxidoreductase, *Giardia lamblia*, DT-diaphorase

INTRODUCTION

The menadione oxidoreductase, or DT-diaphorase, is an enzyme with the molecular formula NAD(P)H and the EC number 1.6.99.2. (QR1), as shown in studies by Cui et al. (1995), Segura- Aguilar et al. (1992), and Tedeschi et al. (1995), catalyses the two-electron reduction of quinones, quinone compounds, and other oxidants using NADPH or NADH as electron donors. Its distribution in animals is extensive. A number of studies have identified the crystal structures of cytosolic enzymes from rats, mice, and humans (Faig et al., 2000; Li et al., 1995). QR1 is made up of two molecules of FAD and two subunits of 273 amino acids. It folds into five β-strands that are parallel to eachother and are bordered on each side by connecting helices. Chenet al. (1994), Cui et al. (1995), Faig et al.(2000), Foster et al. (2000), Li et al. (1995), and Tedeschi et al.

Associate Professor, HOD1,2, Assistant Professor2, Associate Professor, HOD3,
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(1995) are among the structural and enzymic investigations that have provided excellent clarity on the reaction mechanism and the residues interacting with FAD and the two substrates. The QR2 homologue has also been extensively studied in tissues (Faig et al., 2000; Wu et al., 1997). This protein, which has 230 amino acids and is 49% similar to QR1, employs N-ribosyl and N-alkyl dihydronicotinamide as its sole and non-phosphorylated nicotinamide derivatives are unaffected by the signature QR1 inhibitors (Foster et al., 2000; Wu et al., 1997). Foster et al. (1999) found that QR2's high-resolution crystal structure was structurally comparable to QR1's, with the exception that QR2 lacked the 47 amino acid C-terminal region.

Neither the natural substrates of QR1 nor its biological function have been fully clarified despite extensive research spanning over 60 years. Several anti-cancer activities, quinone detoxification, and vitamin K metabolism have all been linked to this enzyme. It also maintains the reduced form of coenzyme Q in membranes. agents (Dinkova-Kostova & Talalay, 2000; Chen et al., 2000). In the presence of NADH and NADPH, a number of oxidoreductases may undergo a one-electron transfer process to decrease quinone molecules to semiquinones. These semiquinones can then reduce oxygen to produce superoxides. On the other hand, when NAD(P)H is present, QR1 may decrease a number of quinone compounds via a two-electron transfer process. Inkova-Kostova & Talalay (2000), Nakamura & Hayashi (1994), Siegel & Ross (2000), and Tedeschi et al. (1995) all point to the DT-diaphorase's potential role in a cellular antioxidant defence system due to its ability to participate in the reduction of multiple compounds through two-electron reduction processes, thus preventing a one-electron redox cycling that produces reactive and harmful oxygen species.We still don't know much about how these cytosolic enzymes are distributed across the biosphere. Diplomonad Giardia lamblia (Weinbach et al., 1980) and Entamoeba histolytica (Weinbach et al., 1977) are two unrelated parasitic unicellular eukaryotes that have reported the activity of an enzyme that may be closely related. Both species lack conventional mitochondria and exhibit a fermentative metabolism (Adam, 1991 ; Brown et al., 1998 ; Martinez-Palomo, 1982 ; Mü ller, 1998)According to Brown et al. (1998), Paget et al. (1989), and Weinbach et al. (1980), these microbes can also absorb oxygen.There is no oxidative phosphorylation or connection to mitochondrial-type cytochrome oxidase during respiration.The amounts of oxygen that these creatures are exposed to may vary greatly.Both inside cells and in the gastrointestinal tissues they come into touch with most likely produce harmful oxygen derivatives.Little is known about their strategies for coping with the possible danger of oxidative stress.Superoxide dismutase (SOD) has not been found in G. lamblia, and neither species contains catalase, glutathione, or glutathione reductase (Brown et al., 1995).One possible mechanism for oxygen detoxification in G. lamblia is an oxidation defence system that includes a cytosolic NADH oxidase, which is thought to work as a terminal oxidase, removing excess reducing equivalents and protecting oxygen-labile proteins by keeping the intracellular environment reduced (Brown et al., 1995, 1996a, 1998). Another mechanism is a membrane- associated NADH peroxidase, which is linked to the observed removal of H O by viable trophozoites (Brown et al., 1995).(Brown et al., 1996b, 1998) and a disulfide reductase that issimilar to thioredoxin and plays a part in managing disulfide redox reactions.There are many different paths that electrons may take, but the exact processes and redox components at work are still amystery.The presence of quinones in both species raises the possibility that they play a role in respiration (Ellis et al., 1994).An active 'DT- diaphorase,' NAD(P)H: quinone-acceptor

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oxidoreductase (EC 1.6.99.2), was thought to be present based on early research showing that NADHand NADPH stimulated respiration.(Wasserbach et al., 1977; Paget et al., 1989 in the year 1980. According to Weinbach et al. (1980), QR1 was shown by the fact that cytosolic fractions exhibited enhanced NADPH oxidation when exposed to 1 mM menadione, and that this process was inhibited by 200 µM dicoumarol. After locating and sequencing a QR gene in G. lamblia, we were able to transfer its expression to Escherichia coli. In this work, we detail the enzyme's characteristics and demonstrate that, while being smaller than its mammalian homologues, it has many of the same biochemical features. Modelling the protein in three dimensions showed that its folding was quite similar, but that it interacted with FAD and its substrates differently.

METHODS

Biological and cellular sample preparations. Axenic culture at 37 ºC in TYI medium (Keister, 1983) was used to cultivate G. lamblia trophozoites (strain WB, supplied by Dr. Stephen Aley of the University of Texas in El Paso, TX, USA). Centrifugation at 700 g for 7 minutes at 4 ºC followed by 10 minutes of cooling on ice was used to extract exponential-phase trophozoites. After being rinsed with phosphate-buffered saline, the pellet was mixed with 250 mM sucrose, 50 mM phosphate buffer pH 7.5, and 10 µg leupeptin ml−′. It was then subjected to five 30-second pulses of ultrasound using a Branson sonifier at 20% duty cycle and output setting 2. The supernatant fraction was either utilised immediately or kept at —20 ºC after centrifugation at 100,000 g for 1 hour at 4 ºC. Activity tests for enzymes. Under aerobic circumstances, a coupled assay was used to detectthe NAD(P)H-dependent decrease of menadione. The absorbance of cytochrome may be monitored at 550 nm (ε = 29·5 mM−¹ cm−¹) as a result of eduction of menadione to menadiol. We used 50 M Tris/HCl pH 7.5, 200 µM NADPH (to act as an electron donor), 1 µM menadione (to act as an electron acceptor), and 30 µM cytochrome c for oureasurements.

The standard combination consisted of 400 µM NADPH, 50 mM Tris/HCl pH 7.3.5, and one of the following for the other electron acceptors that were tested: A solution containing either 50 µM methyl red (MT), 0.5 mM 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), or 0.15% potassium ferricyanide (DCIP) is required. The addition of the enzyme started the reactions, and the reduction of the acceptor was monitored spectrophotometrically at several wavelengths: 600 nm for DCIP (ε = 21·5 mM−¹ cm−¹), 410 nm for potassium ferricyanide (ε = 1 mM−¹ cm−¹), 610 nm for MTT (ε = 11·3 mM−¹ cm−¹), and 436 nm for MT (ε = 18 mM−Œ cm−¹). We defined one unit of enzyme activity as the amount of product generated per minute per milligramme of protein. Where applicable, means are shown ±sd, and all determinations were performed at least three times.

Molecular cloning is that process. Curiously, whenstudying genes related with microtubules, a gene encoding a putative NADPH: quinone oxidoreductase from G. lamblia was identified. The whole glQR1 coding area was amplified by polymerase chain reaction (PCR) using genomic DNA from G. lamblia as a template and the oligonucleotides glQR1-4F (sense: 5'-GACGACGACAAGGGATCCACATCGTCCT
C
- TATTACTCC-3') and glQR1-5R (antisense: 5'-GGACAC-
AGACCCGGGTTACTCGAAGAGCTTCAGG
T
AGCT-3'), according to the available sequence. Sites highlighted indicate that the sense primer added a BamHI site just before the ATG codon and the antisense primer added a SmaI site just after the gene's stop codon. The 500 bp product, which included the whole glQR1 coding area, was then putintopCR2.onevector by the use of an Invitrogen TA Cloning Kit. Before being subcloned into plasmid pQE-32 (Qiagen), clones carrying the glQR1 insert were PCR confirmed and digested with BamHI and SmaI to liberate the glQR1-coding insert. A plasmid-encoded histidine stretch was inserted in-frame

with the glQR1 gene, which was then controlled by an IPTG-inducible lac promoter. Prior to assembly using the Seqman programme of the Lasergene package (DNASTAR), the nucleotide sequence of this construct was confirmed using native cytoplasmic proteins, the recombinant protein was subjected to Ni-NTA resin purification.

Analysis of sequences and modelling of proteins. According to Altschul et al. (1997), the blAST network service was used to search the protein database on the server of the National Centre for Biotechnology. Using the ED programme of the MUST package, the deduced amino acid sequence was manually modified after alignment with similar sequences (Philippe, 1993). Protein Database entries for DT-diaphorase QR1 (file 1QRD), QR2 (files 1QR2 and 2QR2), and DT-diaphorase QR1 (files 1D4A and 1DXO) in humans, mice, and rats were used. In order to generate glQR1 three-dimensional models, these structures served as references inside the programme MODEllER V.4 (Sali & Blundell, 1998 ; Sanchez & Sali, 1997), which made use of comparative protein modelling to meet spatial constraints. Multiple alignments of glQR1 with known structural QRs were fed to MODEllER.

Catalytic properties

With either NADPH or NADH as reductant, the purified recombinant protein reduced menadione and naphthoquinone, with specific activities of 93·53±6·56 and 88·87± 0·96 µmol min^{-1} mg^{-1} and 18·28±1·46 µmol min^{-1} mg^{-1}, respectively. Several benzoquinone derivatives, including 2-hydroxy-1,4-naphthoquinone-1,4-benzoquinone (BQ), 2,6-dimethyl-BQ, and 2,3-dimethoxy-5-methyl-BQ, did not demonstrate any action (data shown).

By changing the concentration of naphthoquinone or menadione while keeping the concentration of NADPH or NADH constant, and vice versa, linear double-reciprocal plots were generated. glQR1 had a comparable and very strong affinity for naphthoquinone and menadione when exposed to primer walking and dye-primer terminator chemistry.

The recombinant protein was expressed and purified. The recombinant plasmid-containing E. coli M15(pREP4) was cultured in LB mediumwith 100 µg ampicillin per millilitre until an optical density (OD) of 0.6 was attained, at 37 ºC.After that, 1 mM IPTG was added and the incubation was kept going for another 6 hours at 37 ºC. In accordance with the guidelines providedby Qiagen for the purification of

The generated glQR1 models, both with and without bound cofactor, were then assessed by PROChECk (Laskowski et al., 1993) and the

E. coli was used to express the promoter region of the pQE-32 vector. The presence of an active histidine-tagged recombinant glQR1 protein was shown by SDS-polyacrylamide gels and enzymic activity determination in IPTG-induced cells carrying the plasmid construct. In contrast, control cells transformed with the plasmid without insert or uninduced cells did not express this protein. Recombinant protein molecular mass was 19 kDa, matching that estimated from nucleotide sequence. Protein band appearance in the gel was associated with QR activity (data not provided) and a very yellow hue. In order to conduct its biochemical analysis, the protein underwent purification by separating it from indigenous bacterial proteins using affinity chromatography in Ni-NTA agarose.

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The InsightII package's hOMOlOgy module (Molecular

alternatively, NADH or NADPH. Two individuals, Kapp and k,

RESULTS

A 495 bp DNA fragment encompassing the full-length coding region of a putative NAD(P)H: quinone oxido-reductase of
G. lamblia was inserted downstream of the electron acceptors were within the same order of magnitude (Table 1). The enzyme was more efficient with menadione than with naphthoquinone, with either NADPH (2·2-fold) or NADH (1·8-fold) as the electron donor. With respect to the electron donor, glQR1 had similar affinities for NADPH and NADH in the presence of naphthoquinone and the reaction was similarly

Table 1. Kinetic parameters of glQR for the electron acceptors

NAD(P): menadione oxidoreductase activity was determined spectrophotometrically by measuring the reduction of cytochrome *c* at 550 nm. Each value is the mean of three to five sets of experiments±sd. Kinetic parameters were determined from Lineweaver–Burk plots generated by using 5–50 μM menadione or naphthoquinone as the electron acceptor. K_i values were determined by varying the menadione or naphthoquinone concentration (5–20 μM) at several fixed concentrations of dicoumarol (0, 0·5, 1, 2, 5 and 10 μM).

Electron donor	Electron acceptor	K_m^{app} (μM)	k_{cat} (s^{-1})	k_{cat}/K_m (μM^{-1} s^{-1})	K_i (μM)
NADPH	Menadione	0·240±0·030	34·83±6·13	145·12	4·12±0·60
	Naphthoquinone	0·59±0·001	39·29±0·68	66·59	5·11±0·91
NADH	Menadione	0·188±0·016	23·07±0·59	121·39	0·95±0·08
	Naphthoquinone	0·437±0·031	29·93±1·78	68·03	1·09±0·12

Table 2. Kinetic parameters of glQR for the electron donors

NAD(P): menadione oxidoreductase activity was determined spectrophotometrically by measuring the reduction of cytochrome *c* at 550 nm. Each value is the mean of three to five sets of experiments±sd. Kinetic parameters were determined from Lineweaver–Burk plots generated by using 5–400 μM NADPH or 5–1000 μM NADH as the electron donor. K_i values were determined by varying the NADPH or NADH concentration (5–400 μM) at several fixed concentrations of dicoumarol (0, 0·5, 1, 2, 5 and 10 μM).

Electron acceptor	Electron donor	K_m^{app} (μM)	k_{cat} (s^{-1})	k_{cat}/K_m (μM^{-1} s^{-1})	K_i (μM)
Menadione	NADPH	30·85±4·70	52·68±1·95	1·71	0·364±0·092
	NADH	549·96±14·50	31·72±5·77	0·06	0·524±0·066
Naphthoquinone	NADPH	33·43±2·16	47·51±3·28	1·42	0·789±0·080
	NADH	44·91±2·73	62·66±5·06	1·40	0·719±0·068

Table 3. Effect of several compounds on glQR1, for different electron acceptors with NAPDH as electron donor

NAD(P): menadione oxidoreductase activity was determined spectrophotometrically by measuring the reduction of the electron acceptors ferricyanide, MTT, MT or DCIP, in the absence or presence of several compounds at the concentrations indicated. Reduction of cytochrome *c* was determined when theelectron acceptor was menadione. All assays were performed at least in duplicate. Values in parenthesesare the specific activities for each electron acceptor (μmol min^{-1} mg^{-1}) determined using the standard assay mixture, and defined as 100 %. ND, Not determined.

Addition	Concn	Relative activity (%) with:

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None		100 (1618)	100 (228)	100 (43)	100 (16)	100 (93)
Dicoumarol	2 μM	46	24	99	87	22
	10 μM	18	14	78	71	2
	20 μM	8	11	52	29	ND
	50 μM	2	9	41	ND	ND
SOD	16 U	105	ND	ND	ND	106
	32 U	96	ND	ND	ND	90–107
Catalase	200 U	109	ND	ND	ND	91
β-Mercaptoethanol	100 μM	145	96	56	100	60
DTT	100 μM	134	113	70	ND	52
N-Ethylmaleimide	50 μM	118	114	65	ND	76
	250 μM	45	90	38	ND	30
l-Cysteine	10 μM	145	99	79	ND	60
	50 μM	132	145	83	ND	9
1,10-Phenathroline	10 μM	32	117	78	37	27
	50 μM	24	44	66	40	9
Quercetin	10 μM	50	27	41	103	83
	50 μM	4	23	18	75	19
Quinacrine dihydrochloride	10 μM	16	77	39	ND	20
	50 μM	16	35	35		5

efficient (Table 2). In contrast, in the presence of menadione, glQR1 had a much higher affinity for NADPH (the K_{app} for NADPH was 18-fold lower than that for NADH) and the reaction was about 29-fold more efficient. With naphthoquinone as electron ac- ceptor, glQR1 became highly specific for NADH. The

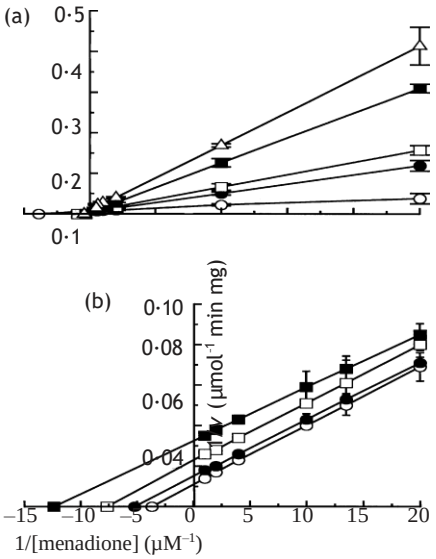

Fig. 1. (a) Competitive inhibition of glQR1 by dicoumarol with respect to the electron donor NADPH in the pres ence of menadione as acceptor. The concentrations of dicoumarol were (○), 0·5 μM (●), 1 μM (□), 2 μM (■) and 3 μM (Q). (b) Uncompetitive inhibition of glQR1 by dicoumarol with respect

to the electron acceptor menadione in the presence of NADPH as donor. The concentrations of dicoumarol were 0(◯), 2 μM (●), 5 μM (□) and7·5 μM (■). The data represent the mean (±SD where appropriate) of two to five determinations.

increase in catalytic efficiency for NADH, in the presence of naphthoquinone compared with menadione, resulted mainly from a much larger effect on the *K*app for NADH

MTT about 3·3 and 4·8 times higher when compared to NADH as a donor (data not shown). Reduction of

Inhibitors and enzyme effectors

The activity of recombinant glQR1 protein decreased by 22 % in the presence of 1 μM dicoumarol, a

The suscep- tibility to

dicoumarol suggested that glQR1 catalysed a diaphorase- or QR1-type oxidoreductase activity. Dicoumarol was a competitive inhibitor (Table 2) with respect to both NADPH (Fig. 1a) and NADH, with both menadione and naphthoquinone. On the other hand, uncompetitive inhibition was observed with menadione (Fig. 1b) and naphthoquinone (Table 1). Dicoumarol at 2 μM inhibited 54 % and 76 % of the ferricyanide and MTT reductase activities, while 20 μM was required for a comparable inhibition of the re- duction of MT and DCIP (Table 3).

Several compounds tested modified the oxidoreductase activity for the several electron

glQR1 was also able to reduce several alternative mitochondrial electron transport inhibitors electron acceptors with NADPH (Table 3) or NADH (not shown) as electron donor. In the presence of NADPH, the reduction rates of the DCIP and MT (specific activities of 16·11±1·38 μmol min^{-1} mg^{-1} and 43·16±1·92 μmol min^{-1} mg^{-1}, respectively) were much lower than the reduction rate of menadione (93·53±6·56 μmol min^{-1} mg^{-1}), whereas they were

QR1 activity in homogenates

G. lamblia homogenates supported the reduction of cytochrome, dependent on the concentration of NADPH, menadione and protein lysate, with a specific activity of 104–294 nmol min^{-1} mg^{-1}. Both menadione and naphthoquinone mediated cytochrome reduction irrespective of whether the hydrogen donor was

the different acceptors did not require an intermediate electron carrier: no significant changes in the rates of the process were observed upon addition of 1–5 μM menadione.

strong inhibitor of the NADPH: quinone reductase reaction, and was essentially abolished at 10 μM.

acceptors in a similar way (activation or inhibition) but to a different extent. FAD, FMN or pyridoxal phosphate (20–50 μM) did not affect the enzyme activity (not shown); however, the flavin antagonists quercetin and quinacrine dihydro- chrloride (10–50 μM) were inhibitory. This suggests that glQR is a flavoenzyme, with the flavin tightly bound, since centrifugation of a protein fraction through a membrane to remove low-molecular-mass species (e.g. FAD/FMN) did not affect glQR1 activity. Catalase and SOD had no effect on the glQR activity.

reductase it enhanced ferricyanide and MTT reduction. Ouabain and chloroquine (10–50 μM) as well as the

KCN and

higher for ferricyanide and MTT (specific activities of 1617±44 μmol min^{-1} mg^{-1} and 288±52 μmol min^{-1} mg^{-1}, respectively). NADPH was a more efficient electron donor, with reduction rates for ferricyanide and

NaN (10–50 μM) had no effect on the glQR activity (data not shown).*

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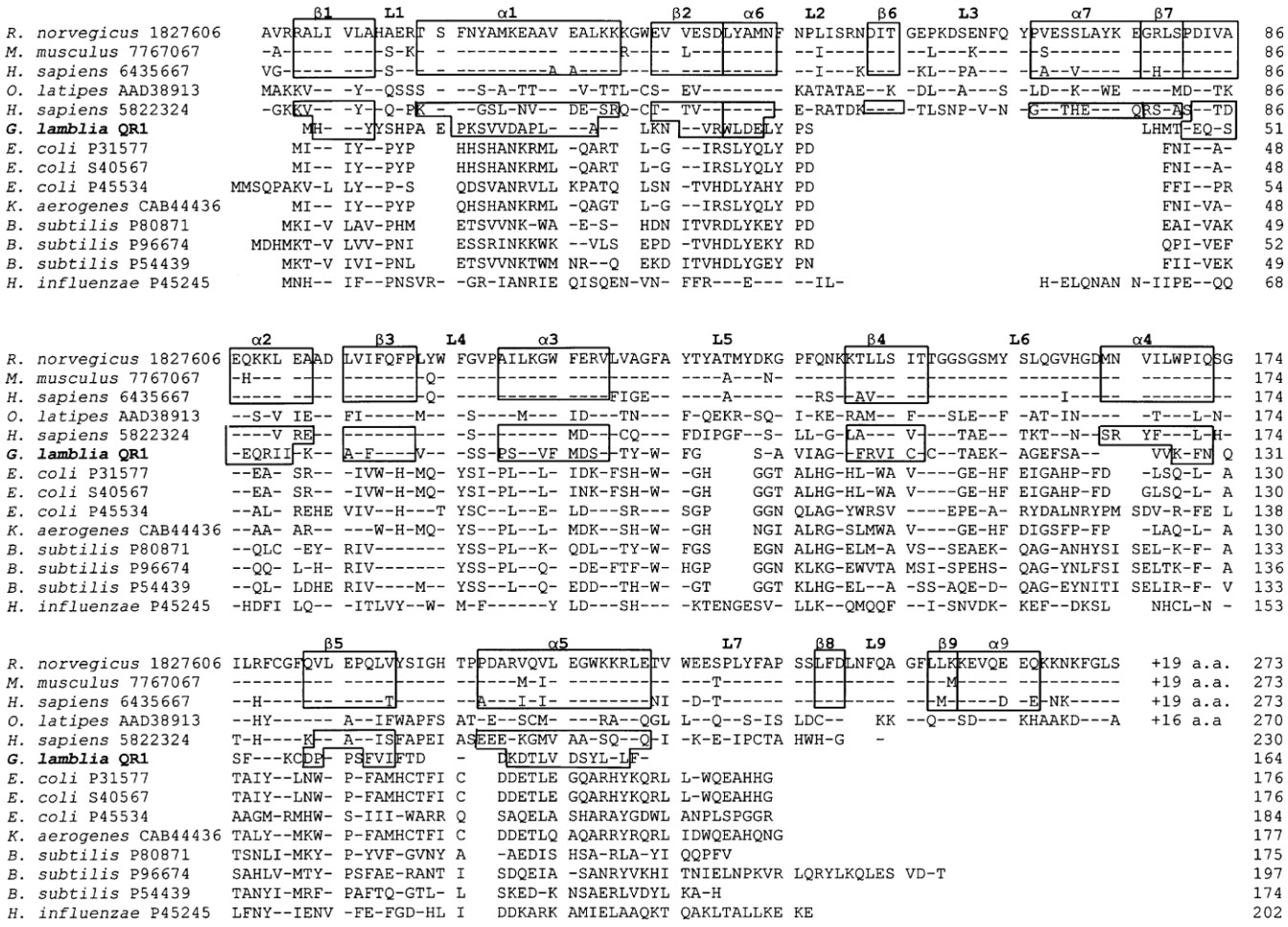

Fig. 2. Amino acid sequence alignment of the glQR1 sequence with several homologous genes from other organisms. Database accession numbers are indicated after the species name. The number of amino acids omitted at the C-terminus of the vertebrate sequences is indicated at the end of the sequences. Dashes represent residues identical to those of the rQR1 sequence; empty spaces represent positions with a gap. The numbering of the amino acid residues for each protein is indicated on the right. Predicted secondary structural elements of the glQR1 sequence and those of the vertebrate enzymes whose X-ray crystallographic structure is available are indicated above the sequence: sheets (β); helices (α) and loops (L). Sheets and helices are also highlighted by boxes. The numbering following the secondary structural elements corresponds to that for rQR1.

NADPH or NADH. Oxygen radical generation in the NADPH: menadione oxidoreductase reaction was investigated by examining the effect of catalase and SOD, which inhibit the superoxide ($O^{\cdot -}$)-mediated reduction of cytochrome *c*. No effect of catalase[1] was observed, while the oxidoreductase activity of the homogenates decreased about 50 % in the presence of SOD (data not shown). This indicated that a fraction of the NAD(P)H: quinone oxidoreductase activity generated $O^{\cdot -}$, possibly by the redox recycling of the menasemiquinone[1] intermediate, which was responsible for cytochrome reduction, while another fraction was able to directly oxidize cytochrome *c*, without $O^{\cdot -}$ with different dicoumarol sensitivities. The partial effect of SOD and dicoumarol on the total activity of *G.lamblia* homogenates suggests that more than one enzymic activity is mediating the oxidation of NAD(P)H with the concomitant reduction of the quinone. However, the proportion of each one cannot be distinguished in the crude homogenates since more than one enzyme contributes to the reduction of the

same electron acceptor.

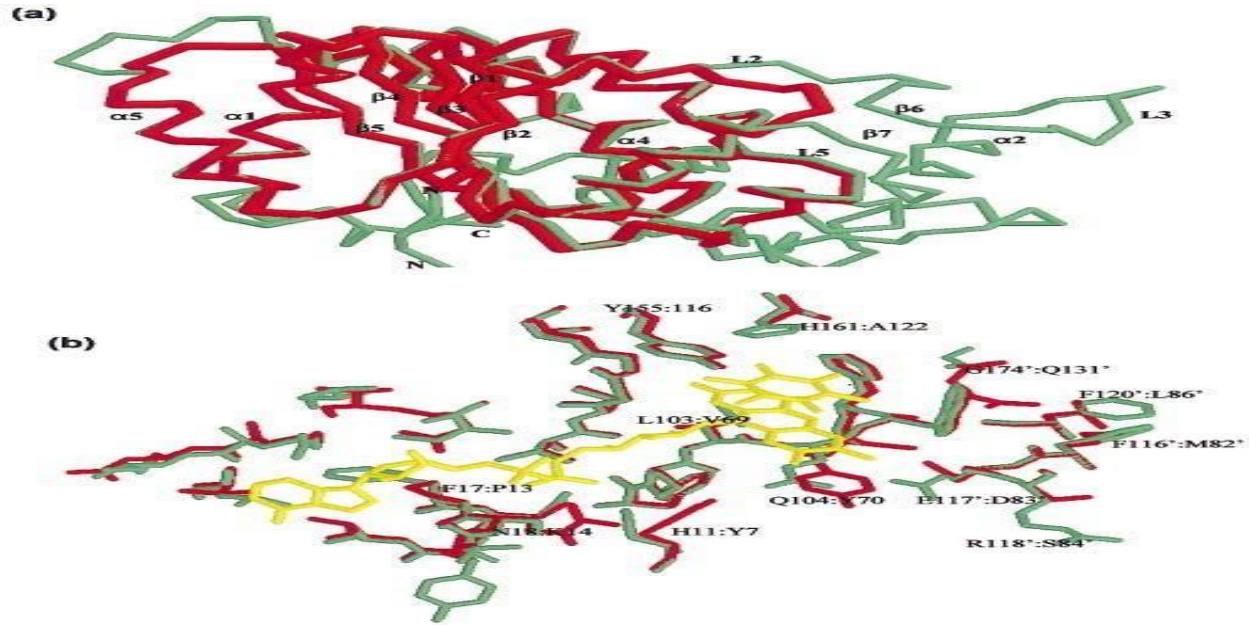

Fig. 3. (a) Superposition of the peptide chain from Cα-atoms of the glQR1 (red) and the hQR1 (green). Helices, sheets and loops are indicated by β, α and L, respectively, followed by a number according to the rQR1 sequence (Li *et al.*, 1995). (b) Superposition of residues involved in binding of the cofactor FAD and the quinone substrate (yellow) and those involved in catalysis for the glQR1 (red) and the hQR1 (green). Substituted amino acids are indicated by the rQR1 amino acid number followed by the glQR1 amino acid number.

Caenorhabditis elegans, Drosophila melanogaster, and Saccharomyces cerevisiae, which are protostome animals. Despite the lack of biochemical characterization, the significant sequence similarity among the eubacterial ORF products suggests that they serve comparable purposes. The sequences are categorised into two distinct size groups. Except for a human paralogue, QR2, which had 230 residues, the ones from vertebrates were 273 amino acid residues long. In contrast, the ones from eubacteria and glQR were much shorter. An analysis of the amino acid sequence (Fig. 2) revealed that the variations in length were mainly caused by an extra 61 amino acids at the C-terminal and a lengthy insertion into the vertebrate sequences (Fig. 2), which match to residues 54-74 of QR1. In the same area, the Haemophilus influenzae ORF also has a shorter insertion. On the whole, glQR1 differed significantly from every other sequence. The C-terminal portion of the sequence (beginning at residue 43) was readily aligned with the

remainder; however, the statistical confirmation of the suggested alignment for the first 42 residues was less clear. Bootstrapping results that were high corroborated the categorization of the sequences into three categories based on their total length (data not shown). While glQR and the eubacterial group share an ancestor, it is situated between the eubacterial and vertebrate groups. Due to the small sample size and high degree of sequence divergence from all other sequences, its true phylogenetic affinities remain unknown.

In order to better understand the enzyme's structure and how it may relate to the similarities and differences in cofactor and inhibitor binding that have been found, three-dimensional models of glQR1 were developed using three X-ray structures of QR1 and QR2. For a more in-depth examination of the active site, which consists of amino acid residues within 6 A˚ [0·6 nm] of the cofactor or substrate, the structures with the lowest energy were chosen. The produced models passed both the protein structure assessment test in Verify 3D and the overall stereochemical criteria used in PROChECk. Potential hydrogen bonding and atomic lengths were determined using the hOMOlOgy. module of InsightII. The secondary structural elements identified in glQR1 are denoted by numerals as described in rQR1 (Li *et al*., 1995). Numbering of the amino acid residues when referred to in the text corresponds to the rat QR1 (rQR1) and glQR1 as indicated in Fig. 2. Amino acid residues on the second chain, contacting the cofactor or substrates, are denoted by the amino acid number followed by a prime (’).

The portion at the end of glQR1 that is N-terminal (residues 1-36) is made up of two β-sheets and an α-helix that overlaps with β1, L1, α1 (which is four amino acids shorter in G. lamblia), and β2 of QR1 (residues 1-40). Instead of the longer portion of QR1 (residues 41-76) that includes loops L2 and L3, helices α6 and α7, and the β-sheet β6, this section of glQR1 is linked to the core area by a 12-amino acid loop (residues 35-46) instead. The portion of glQR1 encompassing residues 47-85 overlaps with the portions of QR1 including α2, β3, L4, and α3 (residues 81-119). A shortened loop region that links residues 87–101 to the C-terminal section of QR1 that contains β4, L6, α4 (which is three amino acids shorter in G. lamblia), β5, and α5 (residues 141-212) overlaps L5 of QR1 (residues 120–140) and completes the connection. glQR1 does not include a section that matches the C-terminal section of QR1, which includes loops L7 and L9, β-sheets β8 and β9, and ñ8 helix. The overall protein fold (Fig. 3a) and the clefts contacting the isoalloxazine ring of the cofactor FAD and the donor/acceptor part of the substrates (the quinone and the nicotinamide moiety of NADPH) are highly conserved, as shown by superimposing the glQR1 models on the crystallographic structures of QR1 (Fig. 3) and QR2. The area of clefts that came into contact with the adenosine ribose part of NAD(P)H and the ribitol, diphosphate, and adenosine moieties of FAD were very different, though, because of changes in amino acids and the removal of certain structural components that made up the clefts (Fig. 3b).

binding of FAD. Y70 overlaps Q104 in the mouse and human QR1 and V69 (of glQR1) overlaps L103 in QR1. Amino acids that anchor the isoalloxazine moiety of FAD through hydrogen bonds in the various glQR1 models are Y70, W71, F72, T108, G110, G111, and Y116. These conserved amino acids structurally overlap with Y104, W105, F106, T147, G149, G150, and Y155 in QR1. Their α-carbon backbones overlap with those of F116', E117', R118', and F120' of QR1, on the other side of the isoalloxazine ring, but their side chains face away from the cleft.

Ribose, diphosphate, and adenosine are all components of FAD, although their respective

locations in the cleft are distinct. H11, F17, N18, and R200 are amino acids that interact with this part of the FAD molecule in QR1. Y7, P13, K14, and K150 are the replacements for these amino acids, respectively. In glQR1, these residues' side chains are either shorter or moved away, but in their native form, their main chains are densely packed around the cofactor and seem to be engaged in no particular interactions.

that separate the FAD molecule from the cofactor and avoid its direct touch. There are no amino acids in glQR1 that interact with FAD, Y67, P68, or E117. These acids are part of L2 and α7.

Quinone binding. G1149 and G150 are amino acids that are present at the entrance of the pocket and superimposed on the W71, F72, F135', G110, and G111 of QR1, respectively. W105, F106, and F178' comprise the interior wall of the pocket. P68', Y128', and H194 are absent, while F92', A122, and Q131' stand in for Y126', H161, and G174', respectively.

NAD(P)H binding. Significant variations can be

the amino acids that mediate interactions between substrates and cofactors. There are morenoticeable variations towards the other side of theactive site. Several interactions with the adenosine ribose portion of the cofactors are absent, mostly because the amino acids responsible for these connections have been deleted. Unlike full-lengthQR1, glQR1 lacks any of the amino acids or structural domains that may change conformation to allow the active site to close or open and mediate various substrate interactions. It seems that glQR1 does not undergo the shielding (from water and perhaps oxygen reactions) that is primarily supplied by the interactions between Y128' and H161 and F232 (Faig et al., 2000). Thisis because the first two amino acids are missing and A122 substitutes for the third. Conversely, at the area superimposed on Y128' of QR1, the glQR1 polypeptide chain (in the shorter loop L5)

observed in the region of the molecule responsible for binding the adenosine moiety of NADPH. This is primarily attributable to the elimination of protein-substrate contacts caused by a 5 amino acid deletion of loop 5 and a truncation of loop L9, which eliminates interactions mediated by L230, N231, F232, and F236. M154, on the second chain Y126', and F178', which are structurally overlapped by glQR1 K115, F92', and F135', respectively, are close to the nicotinamide moiety and have the ability to generate hydrogen bonds or van der Waals interactions. In the same pocket, you may find the amino acid stretch TTGGS, which is overlaid by TCGGT, which is residues 108-111 of glQR1. This stretch is thought to be a component of the NADH-binding site (Li et al., 1995). Y128' and H194 do not overlap with any residues.

Overall structure of the active site. In comparison to QR1, half of the active site in glQR1 is preserved; this is the area where the enzymic hydride transfer occurs and where the isoalloxazine ring of FAD, quinone, and the nicotinamide component of NADPH are located. It is a primary source

began to bend away from the cofactor surrounding F92 (which overlaps Y126' of QR1). H194 in the shorter α5 helix is also in a similar situation. A broader entry to the active site in glQR1 is created by the lack of H194, in conjunction with the absence of a residue overlapping P68'. Y155 of QR1 is preserved in glQR1 and overlaps Y116, making it one of the two amino acid residues that are thought to directly engage in the charge relay that occurs in the necessary two-electron transfer process. Nevertheless, QR1's H161 isused A122 instead of glQR1 as it won't work withthe proposed charge relay. For a mutant, H161 is both an enzyme and a catalytic reaction cofactor (kcat). hH161Q represents only 8% of the hQR1 wild-type allele, yet it Menadione's two-electron transfer reduction should not be interrupted

DISCUSSION

An active G. lamblia NADPH:menadione oxidoreductase has been cloned and expressed by our group. We can conclude this based on two pieces of evidence. To start, known QR genes in animal tissues have a high degree of similarity with the G. lamblia gene's amino acid sequence. Finally, a catalytically active QR with kinetic characteristics and inhibitor susceptibility similar to other species' QR1s was encoded via a recombinant plasmid encoding glQR1 and transfected into E. coli. It seems that the catalytic processes used by the two enzymes are similar, as the most conserved part of glQR1 covers the same area as QR1, which contains several amino acid residues involved in substrate and cofactor binding and catalysis. Dicoumarol had no effect on the enzyme. Since SOD had no effect on glQR1 activity, superoxide formation in the process was ruled out, and menadione was reduced by two electrons.

Despite its shorter length, glQR1 is a genuine QR from a biochemical standpoint. As QR1, it defines a minimum structure that may enable QR activity and includes most of the secondary structure components. The structural changes that occur in QR1 following binding of NAD(P)H are the most noticeable, and glQR1 is missing loop 9 and a portion of loop L5. These variations and changes to amino acids at the active site's entrance provide a larger cleft, which may not be flexible enough to adjust the interactions with the cofactors or substrates suggested for QR1. Nevertheless, glQR1's ability to use a wide variety of electron acceptors demonstrates that its active site can tolerate molecules of different sizes and structures. It is evident from the crystal structures that the C-terminal region of QR1 helps the cofactor bind to the pyrophosphate-ribose adenine. The fact that glQR1 may use NAD(P)H despite having its C-terminal domain cut at residue 212 suggests, however, that these interactions do not seem to distinguish electron donor specificity. It is worth noting that QR2, which is similarly missing the C-terminaldomain.

Like all known QR1s, glQR1 has a substrate selectivity and is susceptible to the same and inhibition by flavoantagonists indicate that G. lamblia is likewise a flavoprotein. The inhibitory impact of flavoantagonists on oxygen absorption and NADPH oxidation in earlier tests indicated a function of flavoproteins in G. lamblia respiration (Paget et al., 1989 ; Weinbach et al., 1980). The physiological role of QR1 has not been the subject of any experiments. Given that NAD(P)H is an electron donor, it's reasonable to assume thatthis enzyme may play a role in nicotinamide metabolism or connect the oxidation of pyrimidine nucleotides to the reduction of a substrate. This inhibitors. The presence of most residues involved in the interaction of the flavin moiety would ensure that glycolysis always has access to oxidised pyrimidine nucleotides. It is yet unknown what physiological function glQR1 plays in G. lamblia metabolism or what substrates it binds to in vivo. Like the enzyme in other organisms, glQR1 can use naphthoquinones and a few artificial electron acceptors to remove oxidants that haven't been identified. This prevents these oxidants from becoming free radicals or forming intermediate species that could react quickly with nucleophiles, such as reduced thiol compounds like cysteine, which would deplete pools of

reduced nucleophiles and nicotinamide. In a broader sense, it may function as a defence mechanism that shields cells from the harmful effects of quinones and other cellular oxidants, such as redox cycling oxidative stress. Eukaryotic organisms other than vertebrates possess this enzyme, as shown by the sequencing and characterisation of the NAD(P)H: menadione oxidoreductase from the amitochondriate G. lamblia. This enzyme's known taxonomic restrictedness is remarkable and requires an explanation, even if biochemical evidence imply the presence of comparable activity in other eukaryotic organisms. The sparse distribution may indicate that there is divergence of homologues that goes beyond what blAST searches can detect. In light of this consideration, it is all the more perplexing that some eubacteria and eukaryotes, including diplomonads and vertebrates, exhibit such remarkable sequence conservation of the enzyme. This conservation may be evidence of horizontal gen

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