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Glyoxalase I of the malarial parasite *Plasmodium falciparum*: evidence for subunit fusion

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Abstract Recombinant *Plasmodium falciparum* glyoxalase I (PfGlx I) was characterized as monomeric Zn^{2+}-containing enzyme of 44 kDa. The K_M value of the methylglyoxal–glutathione adduct is 77 ± 15 μM, the k_{cat} value being 4000 min^{-1} at 25°C and pH 7.0. PfGlx I consists of two halves, each of which is homologous to the small 2-domain glyoxalase I of man. Both parts of the *pfglx I* gene were overexpressed; the C-terminal half of PfGlx I was found to be a stable protein and formed an enzymatically active dimer. These results support the hypothesis of domain-swapping and subunit fusion as mechanisms in glyoxalase I evolution.

Key words: Glutathione; Glyoxalase; Malaria; Methylglyoxal; *Plasmodium falciparum*

1. Introduction

The cytosolic glyoxalase system comprises two enzymes, glyoxalase I (Glx I; EC 4.4.1.5) and glyoxalase II (Glx II; EC 3.1.2.6), and converts toxic 2-oxoaldehydes into 2-hydroxycarboxylic acids, using reduced glutathione (GSH) as a coenzyme (see [1] for review and references therein). Glx I belongs to the VOC metalloenzyme superfamily [2,3] the members of which contain a paired βαββ motif providing for a metal coordination environment [4–6]. Human Glx I is a zinc-dependent homodimeric enzyme. Each subunit consists of two similar domains and appears to have arisen from a gene duplication event [4]. In yeasts [7] and plants [4,8,9], however, glyoxalases I were detected that are doubled in size and thus consist of four homologous domains indicating a further gene duplication with an additional gene fusing event. This hypothesis was supported by studies on yeast Glx I which included modelling, mutagenesis, and functional analysis and suggest that the enzyme has two active sites contained in a single polypeptide [10].

The malarial parasite *Plasmodium falciparum* is responsible for more than 2 million deaths per year. Due to increasing resistances against the presently available drugs, new therapeutic approaches directed against novel targets are urgently required [11–13]. The glyoxalase detoxification system is of particular importance to organisms largely depending on glycolytic energy production such as tumor cells [14] and malarial parasites. *P. falciparum* consumes more than 100-fold more glucose than its host erythrocyte, and correspondingly, has to cope with large quantities of the toxic by-product methylglyoxal. In erythrocytes infected with *P. falciparum* the formation of D-lactate from methylglyoxal was found to be increased by a factor of 30. Inhibitors of Glx I have been shown to exhibit antiproliferative effects on malignant cell lines as well as on parasites [15,16]. *S-p*-Bromobenzylglutathione ethyl diester for instance inhibits the growth of malarial parasites in vitro with an IC_{50} of approximately 5 μM 6 h after exposure [1,17]. Taken together, these facts render the glyoxalase system of *P. falciparum* a promising target for the development of novel antimalarial drugs [18–21].

Here we provide first insight into the malarial glyoxalase system and substantiate the hypothesis that large glyoxalases, like the protein from *P. falciparum*, evolved from small homodimeric glyoxalases by a second gene duplication event.

2. Materials and methods

2.1. Materials

All chemicals used were of the highest available purity and were obtained from Roth (Karlsruhe, Germany), Merck (Frankfurt/M., Germany) or Sigma/Aldrich (Steinheim, Germany). The expression system QIA-express was purchased from Qiagen (Hilden, Germany). Sequencing reactions were carried out on an ABI Prism 310 Genetic Analyzer.

2.2. Molecular biology

The complete open reading frame of a Glx I-like gene was identified on chromosome 11 by online screening of the *P. falciparum* genome sequencing project (www.ncbi.nlm.nih.gov/Malaria/plasmodium-blcus.html; [22]). Two homologous primers – introducing a *Bam*HI and a *Hin*dIII restriction site (underlined) – were derived from this gene: N-terminal PfGlxf1 5′-CGCGGGATCCGCACAAGAAATATCAAATTTAG-3′, C-terminal PfGlxr1 5′-CGCGAAGCTTTTATTTTGCAATAAATGAAGTG-3′. The polymerase chain reaction (PCR) using a gametocyte cDNA library as template was carried out with Taq polymerase (3 min at 94°C; 94°C, 30 s; 50°C, 45 s; 72°C, 90 s; 30 cycles; 72°C, 4 min). The derived fragment of correct size was cloned into pQE30 for sequencing and overexpression. The 5′ and 3′ halves of the *P. falciparum* Glx I (PfGlx I) gene were subcloned analogously.

The *Escherichia coli* strain M15 was used for overexpressing the three inserts at 37°C. The recombinant proteins were purified over a nickel–nitrilotriacetic acid column and concentrated. Protein concentrations were determined at 280 nm on the basis of the calculated respective millimolar absorption coefficients.

2.3. Enzyme assays

Glx I activity was determined from the rate of formation of the thiol ester *S*-D-lactoylglutathione (SLG; $\varepsilon_{240\,nm} = 3.37\ mM^{-1}\ cm^{-1}$)

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[23]. The PfGlx I *standard assay mixture* was experimentally defined as follows: 100 mM potassium phosphate, 100 mM KCl, pH 7.0, 2 mM methylglyoxal, and 1 mM GSH, preincubation at 25°C for 15 min, start with Glx I. For determining the specific activity of Glx I, $NiCl_2$ (20 μM) or $ZnCl_2$ (250 μM) was added to the standard assay mixture in order to guarantee saturation of the enzyme with an activating divalent cation.

2.4. *Cultivation of* P. falciparum

Intraerythrocytic stages of two different chloroquine resistant *P. falciparum* strains (K1, FCR3) were cultured in vitro according to Trager and Jensen [24]. The parasites were synchronized and harvested, and a cell extract was prepared as previously described [25].

2.5. *Immunoblotting*

Rabbit antiserum raised against recombinant PfGlx I was obtained from BioScience, Göttingen, Germany. Protein samples were subjected to 12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and blotted on a polyvinylidene difluoride membrane using a semidry blot procedure. As a second antibody, peroxidase-conjugated swine anti-rabbit immunoglobulins (Dako Diagnostika, Hamburg, Germany) were used.

2.6. *Metal ion analysis*

Zn^{2+} and Ni^{2+} ions were determined by atom absorption spectroscopy (Dr. V. Muntean, Seelig Analytical Laboratories, Karlsruhe, Germany).

For fully saturating the enzyme with metal ions, 7 μM Glx I containing 0.63 zinc ion per molecule in 100 mM potassium phosphate of pH 7.0 and 4°C was incubated overnight with 100 μM $ZnCl_2$ and then exhaustively dialyzed against 1 μM $ZnCl_2$ in the same buffer. Subsequently, the protein content of the retentate was determined, and Zn^{2+} concentrations were assessed by atom absorption spectroscopy. In an analogous experiment $NiCl_2$ was used instead of $ZnCl_2$.

3. Results and discussion

3.1. *In silico analyses and structural considerations*

Alignments of the deduced amino acid sequence of PfGlx I with the respective enzymes from other species indicated identities of up to 43% (see Fig. 1). Furthermore, the enzyme has twice the size of 2-domain glyoxalases for which Glx I from man is the most prominent example. In the homodimeric hu-

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Pf      -------MAQEISNLV-KKYN---VTWQQTMLRIYDPKETVEFYEKNFGMINIHTYHFNE 49
Py      -------MEDKKGLILGKKYN---VTWQQTMLRINNPKETVEFYEKNFGMINIHTYHFNE 50
Ag      ------------------LQN---FLFQQTMYRIKDPRASLPFYNEVLGMNLLCKLDFPE 39
Sc      ----MSTDSTRYPIQIEKASNDPTLLLNHTCLRVKDPARAVKFYTEHFGMKLLSRKDFEE 56
At      AESGKAAQATTQDDLLTWVKND-KRRMLHVVYRVGDMDRTIKFYTECLGMKLLRKRDIPE 122
                            *       :.  *: :   :: ** : :**  :   .: *

Pf      YNFSLYFLITPPYDEEERKKLPEPNTKESEKYLWNLNTVCLELTYNHNSQ---EKLSNGN 106
Py      YNFSLYFMITPPYDEDERKKLPKPNTKESEEYLWNLNTVCLELTHNHNSN---ETLSNGN 107
Ag      AKFSLYFMG---YEDIANQ---PSDRKECVQWAMSR-KGTLELTHNWGTENDAEFKYHNG 92
Sc      AKFSLYFLSFP-KDDIPKNKNGEPDVFSAH--------GVLELTHNWGTEKNPDYKINNG 107
At      EKYTNAFLGYGPEDSH----------------------FVIELTYNYGVD---------- 150
         :::  *:   . :.   ..   ..  .. .   . ..  :***:* . :   .    ..

Pf      NEN-DRGFGHIAFNCNDVIEQCDNLFKK--NVKFHKLPHETKMKTIGFALDPNNYWIEIV 163
Py      NEN-DKGFGHIAFNCENVTEFCDYLFKKK-NVKFHKLPHETKMKTIGFALDPNNYWIEIV 165
Ag      NSD-PRGYGHIGIMVPDVKKACER-FDRL-GVEYVKRPDEGRMKGLAFIKDPDGYWIEIF 149
Sc      NEEPHRGFGHICFSVSDINKTCEELESQ--GVKFKKRLSEGRQKDIAFALDPDGYWIELI 165
At      KYDIGAGFGHFGIAVDDVAKTVELVKAKGGKVSREPGPVKGGKTVIAFIEDPDGYKFELL 210
        : :   *:**: :   :: :  :    :   *.      :   . :.*  **:.* :*:.

Pf      KRSNQ-VKWKNYKNITNFSQTMIRVKNPEKSLYFYIHILGMKLIHVKHCS--DFSLYFLK 220
Py      KRSSE-VKWKNNKNITNFSQTMIRVKDPKKSLYFYIHILGMKLIYTKHT---DYSLYFLK 221
Ag      NASTN-FLFQ---------QTMYRIKDPRASLPFYNEVLGMNLLCKLDFPEAKFSLYFMG 199
Sc      TYSREGQEYPKGSVGNKFNHTMIRIKNPTRSLEFYQNVLGMKLLRTSEHESAKFTLYFLG 225
At      ERGPT---------PEPLCQVMLRVGDLDRAIKFYEKAFGMELLRTRDNPEYKYTIAMMG 261
          .       . .      :.* *: :   :: ** . :**:*:   .    .::: ::

Pf      SNYACAENNKEMIEDQSNKNTNEIYDFNSLKNSYQTDEDYENFKQSWEPVLELTHNHGTE 280
Py      SSYDNNDNNS--LNYQNSENSD--YNFDLLKNSFQSNENYENFKSSWEPVLELTHNHGTE 277
Ag      --------------------------YEDIANQPSDRKECVQWAMSRKGTLELTHNWGTE 233
Sc      --------------------------YGVPKTDS---------VFSCESVLELTHNWGTE 250
At      ------------------------------------------YGPEDKFPVLELTYNYGVT 280
        :.    .....  . .....:..  .     .. .  ..      .   .****:* *.

Pf      DDDNFSYHNGNTEPRGFGHIGFLVNDLENYCKELETLN---VTFKKKVTEGLMKNIAFIY 337
Py      NDENFAYHNGNTEPRGFGHIGFLVDDLQSYCKELESLN---IPFKKKINEGLMNNIAFIY 334
Ag      NDAEFKYHNGNSDPRGYGHIGIMVPDVKKACERFDRLG---VEYVKRPDEGRMKGLAFIK 290
Sc      NDPNFHYHNGNSEPQGYGHICISCDDAGALCKEIEVKYGDKIQWSPKFNQGRMKNIAFLK 310
At      -----EYDKGN----AYAQIAIGTDDVYKTAEAIKLFGG-KITREPGPLPGISTKITACL 330
        .. .  *.:**:.. .:.:* :   *    .: :.      :        *  . ::

Pf      DPDNYVIELIQRDTSFIAK- 356
Py      DPDNYLIELVQRGTSFDPK- 353
Ag      DPDGYWIEIF---------- 300
Sc      DPDGYSIEVVPYGLIA---- 326
At      DPDGWKSVFVDNIDFLKELE 350
        ***.:   ..
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Fig. 1. Alignment of monomeric four-domain glyoxalases I. Residues contributing to GSH binding in the human enzyme [4] are boxed. Metal-binding residues are shadowed. Due to gene duplication these residues are present two times (see also Fig. 2). The *Arabidopsis* protein (identified in this report) is predicted to possess a long N-terminal plant-specific signal sequence which is not shown here. GenBank accession numbers are given in parentheses: Ag, *Anopheles gambiae* str. PEST (EAA00341); At, *Arabidopsis thaliana* (AAL84986); Pf, *Plasmodium falciparum* (AF486284, this paper); Py, *Plasmodium yoelii* (EAA18062); Sc, *Saccharomyces cerevisiae* (CAA67622).

man Glx I [4,26] the active site is located at the dimer interface (see Fig. 2B) where side chains from both subunits interact with GSH and the metal ion, which is zinc in Glx I proteins from most organisms but nickel in the *E. coli* enzyme [27].

In yeasts [7], a number of plants [4,8,9] and *P. falciparum* (this report) Glx I consists of four domains. Further in silico analyses enabled us to identify four-domain-type Glx I also in other *Plasmodia* (*P. yoelii*, *P. vivax*, *P. chabaudi*) and in insects, including the malaria mosquito *Anopheles gambiae*. As

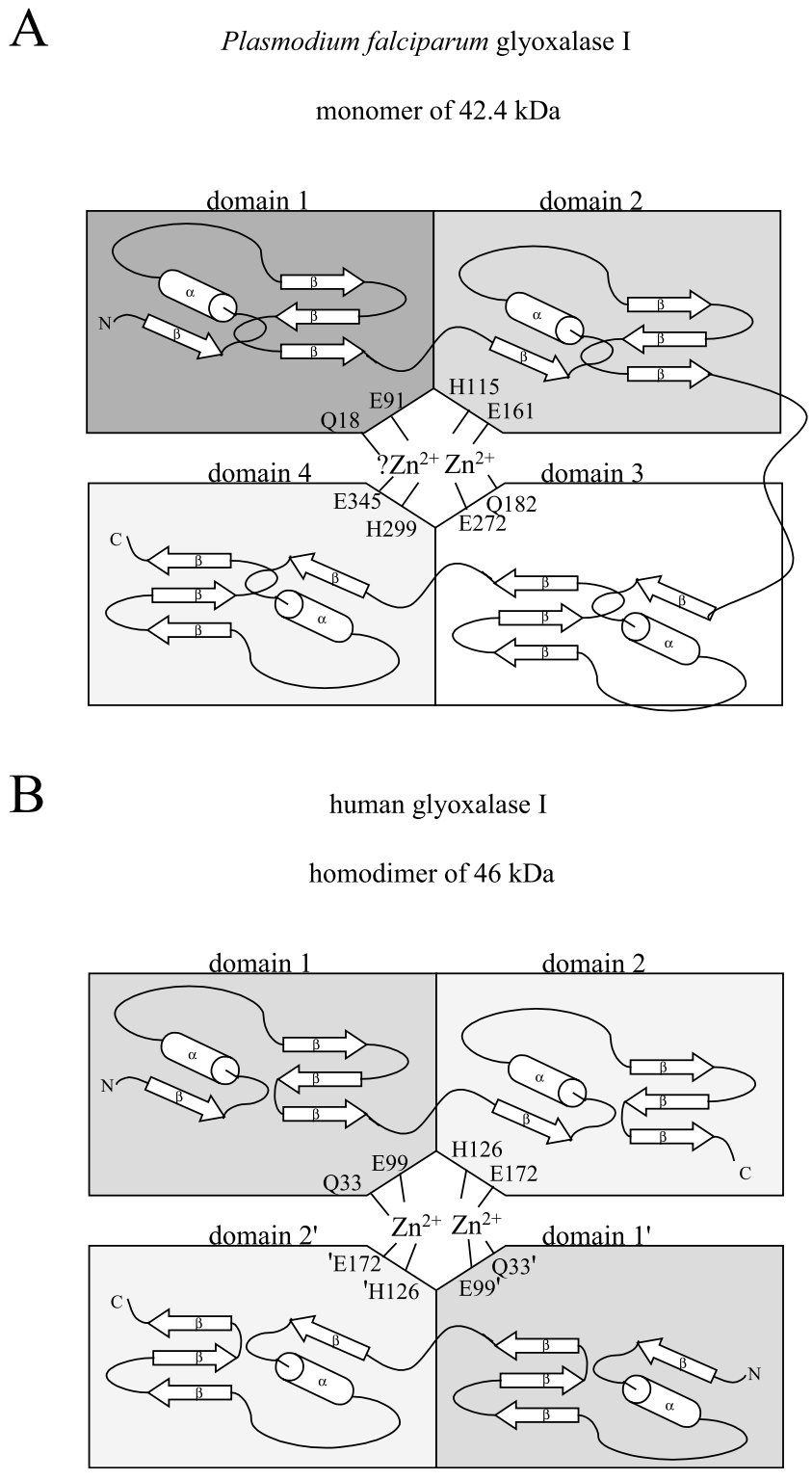

Fig. 2. Schemes of monomeric PfGlx I and homodimeric human Glx I. The βαβββ structure motif is indicated [4], residues involved in metal binding (derived from crystallographic data for human Glx I and derived from secondary structure predictions and alignments in the case of PfGlx I) are numbered according to the sequences of the two proteins. The C-terminal fragment of PfGlx I probably forms a dimeric structure like human Glx I.

indicated in Figs. 1 and 2, the N-terminal (residues 1–172) and the C-terminal part (residues 173–356) of PfGlx I are highly homologous to each other as well as to human Glx I. The residues contributing to glutathione binding in hGlx I correspond to R22/R186, F52/F214, N95/N276, and F151/F335 in the two halves of PfGlx I. The catalytic loop in the human enzyme which is essential for substrate binding and product release [28,29] is conserved in both PfGlx I fragments (see Fig. 1). Based on the putative structure of PfGlx I (Fig. 2A) and the homology with hGlx I [30], two Zn^{2+}-binding sites can be identified in PfGlx I. They are represented by Q18, E91, H299, and E345 for one metal ion and by Q182, E272, H115, and E161 for the second one. It is noteworthy that one metal-binding site is contributed by domains 1 and 4 and the other one by domains 2 and 3 of PfGlx I (Fig. 2A).

All residues representing the binding sites for glutathione and zinc ions, respectively, as well as the catalytic loop of human Glx I are conserved in either half of PfGlx I (Fig. 1). Secondary structure prediction [31] and modeling of the tertiary structure of PfGlx I (Swiss Prot, [32]) confirmed these similarities among the three structures.

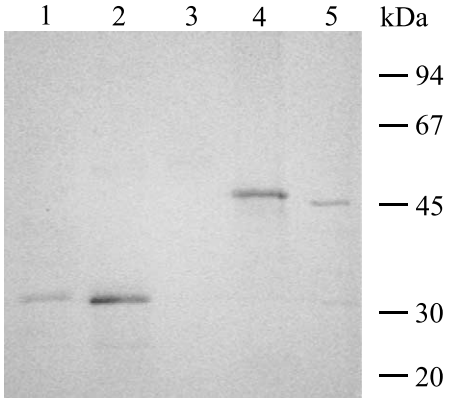

Fig. 3. Western blots of PfGlx I and its fragments. Lane 1: recombinantly expressed N-terminal fragment of PfGlx I (200 ng); lane 2: recombinant C-terminal fragment of PfGlx I (200 ng); lane 3: empty; lane 4: recombinant PfGlx I (150 ng); lane 5: extract of *P. falciparum* strain FCR3 (11 μg total protein). The molecular mass standard is given on the right-hand side. Due to the His-tag, the recombinant enzyme is 1–2 kDa larger than the authentic protein present in parasite extracts.

3.2. Recombinant PfGlx I

PCR amplification, cloning and sequencing of the putative *glx I* gene of *P. falciparum* resulted in a nucleotide sequence which was in full agreement with the gene (1071 bp) of the genomic database (Table 1).

The gene was overexpressed in *E. coli* with a yield of 5 mg l^{-1} cell culture ($OD_{600} = 2.2$). The hexahistidyl-tagged recombinant protein of 43.6 kDa was purified over Ni–NTA agarose and proven to be pure and of correct size by SDS–PAGE and Western blot analysis (see Fig. 3). As shown by gel filtration over a calibrated Sephadex G-200 column PfGlx I is present and active as a monomeric protein (Table 1).

PfGlx I was found to catalyze the intramolecular disproportionation of the hemithioacetal formed by methylglyoxal (2 mM) and glutathione (1 mM) to SLG. A pH profile carried out in 100 mM potassium phosphate, 100 mM KCl indicated an optimum at pH 7.8. Since the spontaneous reaction between GSH and methylglyoxal also increased with increasing pH and in order to enable comparability with previous studies all further assays were carried out at pH 7.0 and 25°C.

For determining the K_M values of the hemithioacetals, the concentration of GSH (30 μM to 1 mM) was systematically varied in the presence of excess (30 mM) 2-oxoaldehyde. The two reactants were preincubated for 15 min to guarantee complete formation of the hemithioacetal [23] before the assay was started with enzyme. The kinetic characteristics of PfGlx I are given in Table 1.

3.3. Inhibition studies

Different known Glx I inhibitors [1,15–17] were studied on the recombinant *P. falciparum* enzyme. PfGlx I was competitively inhibited by the physiologic nitric oxide carrier *S*-nitrosoglutathione ($K_i = 190$ μM) which is known to reach high levels in cerebral malaria, by methylglutathione ($K_i = 170$

Table 1
Characteristics of PfGlx I

Accession number	GenBank™ AF486284
Location in *P. falciparum* genome	Chromosome 11
Genomic DNA numbering	bp 527107–528177
	GenBank™ AL034558
mRNA	1071 bp (with start and stop codon)
Amino acids	356
Molecular mass	42.3 kDa
Molecular mass (with His-tag)	43.6 kDa
Isoelectric point (without His-tag)	5.84
ε at 280 nm	56.8 mM^{-1} cm^{-1}
Bound metal ion	Zn^{2+} and/or Ni^{2+}
pH optimum	7.8
Specific activity	90 U mg^{-1}
k_{cat}	4000 min^{-1}
K_M for glutathione–methylglyoxal hemithioacetal	77 ± 15 μM
k_{cat}/K_M	0.9×10^6 M^{-1} s^{-1}
K_M for glutathione–glyoxal hemithioacetal	580 ± 40 μM
K_i of *S*-nitrosoglutathione	190 μM
K_i of methylglutathione	170 μM
K_i of *S*-*p*-nitrosobenzylglutathione	60 μM
Activity of the C-terminal fragment	3.1 U mg^{-1}
Activity in *P. falciparum* trophozoite extracts	70–140 mU mg^{-1} protein

Down to the line 'ε at 280 nm', the data were deduced from the DNA sequence. The experimental data from 'bound metal ion' to the 'K_i values' refer to the purified recombinant His-tagged protein. The activity in extracts from isolated parasites represents the authentic enzyme.

μM), and by *S*-*p*-nitrobenzylglutathione ($K_i = 60$ μM). These data represent a basis for further inhibitor development aiming at specific inactivation of the parasite enzyme.

3.4. Metal binding to Glx I

As determined by atom absorption spectroscopy, isolated recombinant Glx I contained zinc and nickel in varying molar ratios: 0.4–1.2 zinc and up to 0.1 nickel ions per monomer, the values varying among different preparations. This indicated that the enzyme was not fully saturated with metal ions. Accordingly, the enzyme preparation with 0.6 zinc ions per Glx I was found to be dose dependently activated by 10 min preincubation with different metal salts. Zinc and Mn ions at 250 μM increased enzyme activity by a factor of 2.7 and 1.5, respectively; 20 μM $NiCl_2$ activated the protein 2.9-fold.

When trying to saturate the protein containing 0.6 zinc ions with Ni^{2+} ions, a Glx I containing 1.3 ± 0.3 nickel and 0.6 ± 0.1 zinc ions was obtained. When saturating with $ZnCl_2$ the resulting protein contained 1.4 ± 0.5 zinc ions and 0.05 nickel ions. These results indicate that the enzyme can bind both Ni^{2+} and Zn^{2+} but that Ni^{2+} does not displace zinc ions. Together with the predicted structure of the protein (Fig. 2) these experimental data furthermore confirmed a metal-binding stoichiometry of 2.

3.5. Glx I in P. falciparum

In extracts from isolated *P. falciparum* trophozoites the specific activity of Glx I was 70 mU mg^{-1} in the K1 strain and 140 mU mg^{-1} in the FCR3 strain. The specific reaction of PfGlx I with rabbit IgG raised against the recombinant protein was demonstrated by Western blotting (Fig. 3).

3.6. Structure duplication

The hypothesis that PfGlx I has evolved from two homologous parts, each of which resembling a functional small Glx, was further addressed using a protein engineering approach. The N-terminal half (comprising residues 1–172) and the C-terminal half (comprising residues 173–356) were recombinantly produced as His-tagged proteins. Western blot analysis of the products resulted in single bands of expected sizes (Fig. 3, left lanes). The N-terminal fragment was poorly soluble and thus remained largely in the cell pellet. In contrast, the C-terminal fragment was purified to homogeneity with a yield of 0.3 mg l^{-1} ($OD_{600} = 1.8$).

In the presence of 100 μM $ZnCl_2$ the specific activity of the C-terminal half was determined to be 3.1 U mg^{-1}, which corresponds to 3.4% of wild-type activity. The K_M for the glutathione–methylglyoxal hemithioacetal was approximately 100 μM, which is very similar to the wild-type. FPLC analysis (gel filtration over a calibrated Superdex 75 column) of the C-terminal fragment in the presence of 100 μM $ZnCl_2$ suggested a dimer/monomer equilibrium, the peaks appearing at 28 and 46 kDa. The assumption that the dimer represents the active species was verified by enzyme assays where the activity was studied as a function of protein concentration in the range of 50–1000 nM C-terminal fragment. The results are consistent with the monomer/dimer equilibrium $2\ ZnM = Zn_2D$, the dissociation constant $K = [ZnM]^2/[Zn_2D]$ being 28 μM. This suggests strongly that the enzymatic characteristics of the C-fragment dimer are similar to those of intact Glx I. The most plausible structure of the dimeric C-fragment is similar to that of Human Glx I (Fig. 2B). Thus the C-fragment of Glx I is suitable for screening tests where we expect to identify structure-dissociating agents as inhibitors of PfGlx I. Taken together, our findings indicate that the ancestor of PfGlx I was active as a homodimer (represented by the dimer of the C-terminal fragment) before gene duplication and fusion gave rise to a four-domain monomer.

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