Allosteric Coupling of Two Different Functional Active Sites in Monomeric Plasmodium falciparum Glyoxalase I*§

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Glyoxalase I (GloI) catalyzes the glutathione-dependent conversion of 2-oxoaldehydes to S-2-hydroxyacylglutathione derivatives. Studies on GloI from diverse organisms such as man, bacteria, yeast, and different parasites show striking differences among these potentially isofunctional enzymes as far as metal content and the number of active sites per subunit are concerned. So far, it is not known whether this structural variability is linked to catalytic or regulatory features in vivo. Here we show that recombinant GloI from the malaria parasite Plasmodium falciparum has a high- and a low-affinity binding site for the diastereomeric hemithioacetals formed by addition of glutathione to 2-oxoaldehydes. Both active sites of the monomeric enzyme are functional and have similar $K_{cat}$ values. Proteolytic susceptibility studies and detailed analyses of the steady-state kinetics of active-site mutants suggest that both reaction centers can adopt two discrete conformations and are allosterically coupled. As a result of the positive homotropic allosteric coupling, P. falciparum GloI has an increased affinity at low substrate concentrations and an increased activity at higher substrate concentrations. This could also be the case for GloI from yeast and other organisms. Potential physiologically relevant differences between monomeric GloI and homodimeric GloI are discussed. Our results provide a strong basis for drug development strategies and significantly enhance our understanding of GloI kinetics and structure-function relationships. Furthermore, they extend the current knowledge on allosteric regulation of monomeric proteins in general.

The ubiquitous glyoxalase system comprises two enzymes that catalyze the sequential glutathione (or in rare cases, trypanothione)-dependent conversion of methylglyoxal and other 2-oxoaldehydes to 2-hydroxyacylcarboxylic acids. In this reaction, rate-determining dehydration of hydrated 2-oxoaldehyde is followed by the spontaneous formation of diastereomeric hemithioacetals between GSH and the 2-oxoaldehyde (Fig. 1A) (1, 2). The first enzyme, glyoxalase I (GloI2; EC 4.4.1.5), isomerizes both hemithioacetals to a single diastereomeric thioester. The second enzyme, glyoxalase II (EC 3.1.2.6), hydrolyzes the thioester, releasing GSH and 2-hydroxycarboxylic acid (see Ref. 3 for review). Thus, GSH acts as a coenzyme and is not consumed in the overall reaction. Despite decades of intensive research, the physiological functions of the glyoxalase system and the sources, toxicities, and potential functions of its substrates are still a matter of debate.

To date, GloI from different organisms can be roughly subdivided into three different groups according to the type of divalent cation bound at the active site and the number of subunits forming the functional enzyme (Fig. 1B). For example, GloI from human and yeast (4) and Plasmodium falciparum (5) prefers Zn$^{2+}$, whereas GloI from several bacteria such as Escherichia coli (6) and Yersinia pestis, Pseudomonas aeruginosa, and Neisseria meningitidis (7) and from the protozoan parasite Leishmania major (8, 9) is optimally activated in the presence of Ni$^{2+}$. GloI from human (10, 11) and E. coli (12) is active as a homodimer. The crystal structures of GloI from human (11), L. major (9), and E. coli (12) show that both subunits contribute residues to both active sites of the homodimer. The N-terminal domain of one subunit interacts with the C-terminal domain of the other subunit (Fig. 1B). Homodimeric GloI consist of four $\beta$ab$\beta$ domains (which appear to have arisen by a gene duplication event) forming an eight-stranded $\beta$-sheet (10). In contrast, GloI from Saccharomyces cerevisiae (13) and the malaria parasite P. falciparum (5, 14) is approximately twice the size of homodimeric GloI and has two potential active sites per monomer (Fig. 1B). An alignment of monomeric (putative) GloI from plants, apicomplexan parasites, and Ascomycetes is shown in supplemental Fig. 9. Frickel et al. (13) demonstrated, by site-directed mutagenesis, that both active sites of GloI from yeast are functional. Studies on P. falciparum (PfGloI) support the hypothesis that a second gene duplication and fusion event occurred during evolution of monomeric GloI (5). No structure of a monomeric GloI has been solved yet, and according to sequence alignments and partial molecular models, it is quite likely that the structure of PfGloI differs significantly from the analyzed structures of dimeric GloI (14). PfGloI might be suited as a target for novel antimalarial drugs (5, 14); however, it is not known which of the potential active sites should be targeted for inhibition.

Traditionally, allosteric effects are explained either by the symmetry model of Monod et al. (15) or by the sequential

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The abbreviations used are: GloI, glyoxalase I; PfGloI, P. falciparum GloI; MOPS, 3-(N-morpholino)propanesulfonic acid; MES, 2-(N-morpholino)ethanesulfonic acid.
model of Koshland et al. (16). These classical models of allosteric effects are restricted to oligomeric proteins. However, allosteric (allosteric = other space) means that action in one part of a molecule causes an effect at another site (see Ref. 17 for review). According to this broader definition, allosteric systems are not restricted to oligomeric proteins because the binding of one ligand might affect another binding site of the same monomeric protein. Depending on whether the effector is identical or non-identical to the substrate, homotropic and heterotropic allosteric effectors are distinguished (17). In the case of monomeric proteins, studies on allosteric regulation are usually restricted to heterotropic allosteric interactions.

In this study, we address the questions of whether both potential active sites of PfGloI are functional, whether they are identical in terms of their kinetic parameters, and whether they act independently. Using site-directed mutagenesis and steady-state kinetic measurements, we show that monomeric PfGloI has two different functional active sites with clearly distinguishable substrate affinities. We suggest that both active sites are able to adopt two different conformations and are allosterically coupled. As a consequence, previous results on monomeric Glo from yeast can be reinterpreted. Our results significantly improve the understanding of GloI kinetics as well as structure-function relationships and extend the knowledge of homotropic allosteric regulation of monomeric proteins in general.

**EXPERIMENTAL PROCEDURES**

**Site-directed Mutagenesis**—Mutations of the PfGloI gene (E91Q, E161Q, E272Q, E345Q, E91Q/E345Q, E161Q/E272Q, E161Q/E345Q, R22E, R22E/E91Q/E345Q, and E161Q/R186/E272Q) were introduced by PCR with *Pfu* polymerase (Promega Corp.) using mutated primers (Table 1) and PfGloI (5) as a template. Methylated non-mutated template plasmids were digested with DpnI (Fermentas), and competent XL1-Blue cells were freshly transformed with the respective plasmid before each expression. Initial expression and purification experiments with PfGloI were carried out as described previously (5, 14) before Tris and phosphate buffers were replaced with MOPS buffers. Bacteria were precultured for ~8 h at 37 °C in medium containing 10 g/liter yeast extract, 5 g/liter peptone, 10 g/liter NaCl, 2 g/liter MgSO$_4$·7H$_2$O, and 10 mM MOPS/NaOH (pH 7.5) supplemented with 50 mg/liter kanamycin and 100 mg/liter carbenicillin. The preculture was added to fresh medium and grown at 30 °C to an absorbance at 600 nm of 0.3. Then, 286 mg/liter ZnSO$_4$·7H$_2$O were added, and the culture was grown for 30 min before induction with 0.3 mM isopropyl β-D-thiogalactopyranoside. Cells from 3 liters of culture were harvested by centrifugation (15 min, 6000 × g) ~14 h after induction and resuspended in 20 ml of buffer containing 10 mM MOPS/NaOH (pH 7.8), lysozyme, DNase I, and ~75 µM phenylmethanesulfonfyl fluoride. Resuspended bacteria were stirred for 1 h on ice, followed by sonication at 4 °C and centrifugation for 30 min at 30,600 × g. The supernatant was applied

**TABLE 1**

| Mutation | Sequence |
|----------|----------|
| E91Q | 5'-GAATACAGTTTGCTGCACTCTACATTATAACCT-3' |
| E161Q | 5'-CTAAATATTGAGTTAATTAAATTAAACACAGT-3' |
| E272Q | 5'-GGAGACCCGCTCTAAATTAAACCACAAACG-3' |
| E345Q | 5'-CCTTTTCTCAAACAATGATTGGAAACCCGTTCTA-3' |
| R22E | 5'-CTTTTTTTCGCTGCTTAAATTATTTATAG-3' |
| R186E | 5'-CAGGGTTCTTAAACCATATTATACCCAAGTTATTT-3' |

expressed in *E. coli* strain M15 (Qiagen Inc.). Competent cells were freshly transformed with the respective plasmid before each expression. Initial expression and purification experiments with PfGloI were carried out as described previously (5, 14) before Tris and phosphate buffers were replaced with MOPS buffers. Bacteria were precultured for ~8 h at 37 °C in medium containing 10 g/liter yeast extract, 5 g/liter peptone, 10 g/liter NaCl, 2 g/liter MgSO$_4$·7H$_2$O, and 10 mM MOPS/NaOH (pH 7.5) supplemented with 50 mg/liter kanamycin and 100 mg/liter carbenicillin. The preculture was added to fresh medium and grown at 30 °C to an absorbance at 600 nm of 0.3. Then, 286 mg/liter ZnSO$_4$·7H$_2$O were added, and the culture was grown for 30 min before induction with 0.3 mM isopropyl β-D-thiogalactopyranoside. Cells from 3 liters of culture were harvested by centrifugation (15 min, 6000 × g) ~14 h after induction and resuspended in 20 ml of buffer containing 10 mM MOPS/NaOH (pH 7.8), lysozyme, DNase I, and ~75 µM phenylmethanesulfonfyl fluoride. Resuspended bacteria were stirred for 1 h on ice, followed by sonication at 4 °C and centrifugation for 30 min at 30,600 × g. The supernatant was applied
to an S-hexylglutathione-agarose column (Sigma), which was equilibrated with buffer containing 10 mM MOPS/NaOH (pH 7.8). The column was washed with 8 column volumes of 10 mM MOPS/NaOH and 200 mM NaCl (pH 7.8), and recombinant enzyme was eluted with 3 column volumes of 10 mM MOPS/NaOH, 200 mM NaCl, and 5 mM S-hexylglutathione (pH 7.8). The eluate was loaded onto a nickel-nitrilotriacetic acid column (Qiagen Inc.), which was equilibrated with buffer containing 50 mM MOPS/NaOH, 300 mM NaCl, and 10 mM imidazole (pH 8.0). Afterward, the column was washed with 8 column volumes of the same buffer. The purified enzyme was eluted with buffer containing 50 mM MOPS/NaOH, 300 mM NaCl, and 125 mM imidazole (pH 8.0). Protein concentrations of the fractions were determined using the Bradford assay (18) with bovine serum albumin as a standard. The purity of the eluate was confirmed by reducing 10% SDS-PAGE (19). Determinations of $k_{\text{cat}}$ and $K_m$ values were performed right after purification, and the proteolytic susceptibility or the pH profiles were analyzed the next day.

**Steady-state Kinetics**—The steady-state kinetics of the recombinant enzymes were monitored spectrophotometrically using a thermostatted Hitachi U-2001 or a Jasco V-550 UV-visible spectrophotometer. To determine $k_{\text{cat}}$ and $K_m$ values at 30 °C, experiments were performed as described previously (14). Initial measurements were carried out in assay buffer containing 100 mM K$_2$HPO$_4$ and 100 mM KCl (pH 7.0) (14), whereas the phosphate buffer was later replaced with buffer containing 50 mM MOPS/NaOH (pH 7.0) (see “Results and Discussion”). Stock solutions of 10 mM GSH (Sigma) and 100 mM methylglyoxal (Sigma) in assay buffer were freshly prepared before each experiment. For a desired concentration of hemithioacetal, the concentrations of methylglyoxal and GSH in the assay mixture were calculated and varied using the equation $K_d = \frac{[\text{methylglyoxal}] [\text{GSH}]}{[\text{hemithioacetal}]}$. The total assay volume was 1 ml. The calculated concentration of free GSH after 5 min of preincubation at 30 °C was 0.1 mM in all assays (14). The calculated initial concentration of the hemithioacetal was 5–500 μM. All reactions were initiated by the addition of enzyme, and the formation of S-D-lactoylglutathione was followed spectrophotometrically at 240 nm ($\varepsilon = 3.37 \text{ M}^{-1} \text{ cm}^{-1}$). The final assay concentration of the wild-type enzyme and mutants was 2.5–15 nM, with the exception of PfGloI(E161Q/E345Q) (up to 0.1 μM).

The kinetic data of the initial reaction velocities were plotted according to Michaelis-Menten, Lineweaver-Burk, Eadie-Hofstee, and Hanes and fitted using the program SigmaPlot 10.0 (Systat Software Inc.). The plots of several PfGloI mutants were biphasic, and as a consequence, two apparent values for $K_m$ ($K_{\text{app1}}$ and $K_{\text{app2}}$) and $k_{\text{cat}}$ ($k_{\text{app1}}$ and $k_{\text{app2}}$) were determined by linear regression. The $K_m$ and $k_{\text{cat}}$ values calculated from the different plots of a single experiment varied by <10%. Hill coefficients ($n_h$) of both phases were determined in a Hill plot by linear regression (using $V_{\text{max}} = [E] k_{\text{app1}}$ to calculate the maximum reaction velocity of mutants with biphasic kinetics). An equation describing the steady-state reaction velocity according to the scheme shown in Fig. 8 was computed using the program ALBASS 1.0 (20).

**RESULTS AND DISCUSSION**

**Generation of PfGloI Active-site Mutants**—Each active site of GloI enzymes contains two highly conserved glutamate residues that are most likely involved in acid-base catalysis during substrate enediolate formation and reproteination (21–23). According to a previously proposed structural model of PfGloI (14), the pairs Glu91/Glu345 and Glu161/Glu272 are these critical residues at the two putative active sites, which we will refer to as sites A and B, respectively (supplemental Fig. 10). To study their role in catalysis, we generated a set of three different mutants at putative active site A and Glu161 and/or Glu272 at putative active site B. In a seventh mutant, both active sites were subjected to mutation by substituting glutamine for Glu161 and Glu272. All of the recombinant proteins were purified to electrophoretic homogeneity (as judged by SDS-PAGE) by two consecutive affinity chromatographic steps (supplemental Fig. 11). The wild-type enzyme and all seven mutants were soluble and stable. The average final yield of the purified proteins

**pH Profiles**—The initial velocities of the formation of S-D-lactoylglutathione were measured at pH values ranging from 5.5 to 9.0 at 25 °C. Stock solutions of 100 mM GSH and 1.0 M methylglyoxal in Millipore water were freshly prepared before each experiment. The calculated initial concentration of the hemithioacetal between methylglyoxal and GSH in the assay was 400 μM, and the calculated concentration of free GSH after 5 min of preincubation was 0.1 mM. Assays were performed using three different buffers containing 50 mM MES (pH 5.5–6.75), 50 mM MOPS (pH 6.5–8.0), or 50 mM Tris (pH 7.4–9.0).

**Gel Filtration Chromatography**—The apparent molecular mass of the wild-type enzyme was studied by gel filtration chromatography on a HiLoad 16/60 Superdex 200 preparation grade column, which was connected to an ÄKTA FPLC system (Amersham Biosciences). The column was calibrated with a gel filtration standard (Amersham Biosciences) and equilibrated with buffer containing 50 mM MOPS/NaOH, 300 mM NaCl, and 10 μM ZnCl$_2$ (pH 7.0). Fast protein liquid chromatography fractions were detected photometrically, and peak areas and $k_{\text{app}}$ values were evaluated using the software UNICORN 4.11 (Amersham Biosciences).

**Proteolytic Susceptibility**—The wild-type enzyme and PfGloI(E161Q/E345Q) were concentrated in a Centricron YM-10 device (Millipore Corp.) and treated with subtilisin (Sigma), and the time-dependent protein degradation in the presence or absence of ligands at 25 °C was analyzed by reducing 15% SDS-PAGE. All protease assays were performed in 50 mM MOPS/NaOH (pH 7.0) and started by the addition of subtilisin (50–200 milliunits/ml). The pH of the assay solutions containing either 20 mM S-hexylglutathione or 5 mM hemithioacetal and 0.1 mM GSH was readjusted before the addition of 10–15 μM PfGloI. GSH and methylglyoxal were preincubated for 5 min without PfGloI to allow formation of the hemithioacetal. Aliquots (20 μl) were withdrawn from the digestion mixture (90 μl) after 0, 1, 5, and 10 min and incubated with 4 μl phenylmethylthanesulfonyl fluoride (final concentration of 17 mM) for 2 min on ice before the addition of 10 μl of SDS-PAGE sample buffer and boiling.
strongly depended on the amino acid replacement and differed between 0.3 nmol \((\text{PfGloI(E161Q/E345Q)})\) and 25 nmol \((\text{PfGloI(E272Q)})\) per liter of \(E. coli\) culture.

Phosphate Buffers Inactivate \(\text{PfGloI}\) Active-site Mutants during Catalytic Turnover but Not During Storage—The standard buffers used to measure \(\text{GloI}\) activity contain (di)hydrogen phosphate for historical reasons (24, 25). In previous studies, recombinant wild-type \(\text{PfGloI}\) was also purified and assayed in (di)hydrogen phosphate-containing buffers (5, 14). However, our initial steady-state kinetic measurements of recombinant \(\text{PfGloI(E91Q)}\) (Fig. 2) and of other mutants (data not shown) revealed that these enzymes were rapidly inactivated during the assay when a phosphate buffer was used. Determination of reaction velocities right after the addition of enzyme was difficult, whereas smaller reaction velocities after 60 s could be accurately reproduced (see, for example, first pair of error bars in Fig. 2, A and B). This observation was surprising because \(\text{PfGloI(E91Q)}\) was stable for several days with only slight loss of activity when stored at 4 °C in buffer containing 50 mM sodium (di)hydrogen phosphate, 300 mM sodium chloride, and 125 mM imidazole (pH 8.0). Inactivation during the assay was decelerated, and the overall reaction velocity was increased by the addition of zinc chloride to the assay mixture or by using an alternative assay buffer containing MOPS instead of phosphate. The addition of zinc chloride to the MOPS-buffered assay mixture did not further increase the activity or slow down the inactivation (Fig. 2, A and B). The wild-type enzyme also had a slightly increased activity in the presence of sodium or potassium chloride (D). Assays were performed with 0.4 mM substrate in 50 mM MOPS/NaOH (pH 7.0). Millimolar salt concentrations are indicated.
buffer (Fig. 2C), and PfGloI(E91Q) had a decreased activity at high concentrations of sodium or potassium chloride (Fig. 2D).

We suggest that an interaction between (di)hydrogen phosphate anions and Zn$^{2+}$ at the active site leads to loss or shielding of the cation during catalytic turnover, resulting in decreased activity and rapid inactivation of PfGloI(E91Q) and other active-site mutants. In the absence of substrate, the zinc cations seem to be protected from an interaction with (di)hydrogen phosphate, explaining the stability of the mutants during storage and pointing to a conformational change during catalysis (see below). As a consequence, the expression and purification protocol was modified (see "Experimental Procedures"), and all following assays for determination of the $K_m$ and $k_{cat}$ values for wild-type PfGloI and mutants were performed in buffer containing 50 mM MOPS/NaOH (pH 7.0) without additional salt (supplemental Fig. 12).

**Analyses of the Steady-state Kinetics Reveal Two Functional Active Sites with Different Substrate Binding Properties**—In contrast to previous reports (5, 14), we found that the steady-state kinetics of PfGloI did not follow typical Michaelis-Menten kinetics. The unusual kinetics were probably overlooked because fewer data points were determined and because the evaluation was based solely on Lineweaver-Burk plots. Our plots of the kinetic data are biphasic for the wild-type enzyme and, to a lesser degree, for the PfGloI mutants. Without postulating a mechanism and a model that results in complex equations, the data were fitted empirically by two straight lines (Fig. 3). Accordingly, two apparent values for $K_m$ ($K_{m1}^{app}$ and $K_{m2}^{app}$) and $k_{cat}$ ($k_{cat1}^{app}$ and $k_{cat2}^{app}$) were determined by linear regression (Table 2). At this point, these values were not attributed to active site A or B. The $K_{m1}^{app}$ and $k_{cat1}^{app}$ values determined at lower substrate concentrations (5–50 μM hemithioacetal) are smaller than the $K_{m2}^{app}$ and $k_{cat2}^{app}$ values determined at higher substrate concentrations (0.2–0.5 mM hemithioacetal). Hill plots (Fig. 3) reveal a Hill coefficient ($n_H1$) < 1 for lower substrate concentrations. The wild-type enzyme and all seven mutants can be divided into four different groups (Table 2). PfGloI(E161Q/E345Q) is almost completely inactivated, whereas $k_{cat2}^{app}$ for the wild-type enzyme is ~2-fold larger than $k_{cat2}^{app}$ for the remaining six glutamate mutants. These six mutants can be further subdivided into two groups depending on $K_{m1}^{app}$, $K_{m2}^{app}$, and the maximum catalytic efficiency ($k_{cat2}^{app}/k_{cat1}^{app}$). In both groups, $K_{m2}^{app}$ is about twice as high as $K_{m1}^{app}$; however, the $K_{m1}^{app}$ values for the two groups differ by roughly an order of magnitude: $k_{cat1}^{app}$ for the wild-type enzyme increases by 5-fold upon replacement of Glu$^{91}$ and/or Glu$^{345}$, whereas $k_{cat2}^{app}$ for the wild-type enzyme decreases by ~5-fold upon replacement of Glu$^{272}$ and/or Glu$^{161}$. As a result, the maximum catalytic efficiency of PfGloI(E91Q), PfGloI(E345Q), and PfGloI(E91Q/E345Q) is 7% of the wild-type enzyme compared with 60% for PfGloI(E272Q), PfGloI(E161Q), and PfGloI(E161Q/E272Q).

Table 2 shows that Glu$^{91}$/Glu$^{345}$ and Glu$^{345}$/Glu$^{161}$ are functionally paired in PfGloI. The requirement of two glutamate residues per active site is in accordance with the current model of the GloI-catalyzed reaction mechanism of both diastereomeric substrates. NMR studies on monomeric GloI from yeast indicated that both diastereomeric hemithioacetals between GSH and methylglyoxal are turned over with identical reaction rates (26). Studies on human GloI suggested that Glu$^{172}$ is required for proton transfer between C-1 and C-2 in both diastereomers, whereas Glu$^{99}$ is required only for deprotonation of C-1 of the R-diastereomer (for details, see Refs. 22 and 23). The slightly increased values of $k_{cat1}^{app}$ for PfGloI(E91Q) and PfGloI(E272Q) compared with $k_{cat1}^{app}$ for PfGloI(E345Q) and PfGloI(E161Q), respectively, might reflect a residual activity of the modified active sites of PfGloI(E91Q) and PfGloI(E272Q) because Glu$^{91}$ and Glu$^{272}$ are probably not required for C-1–C-2 proton transfer of the S-diastereomer, whereas Glu$^{345}$ and Glu$^{161}$ are essential for the turnover of both substrates. Even though the modified active sites of PfGloI(E91Q) and PfGloI(E272Q) might still have a residual activity with the S-diastereomer, they should be strongly inhibited in the assay because the R-diastereomer becomes a (competitive) inhibitor. Taking together our data and previous studies on GloI from other organisms, we suggest that wild-type PfGloI also converts both diastereomeric hemithioacetals between GSH and methylglyoxal with identical reaction rates. We conclude from the different types of mutants that PfGloI has two functional active sites and that Glu$^{91}$/Glu$^{272}$ and Glu$^{345}$/Glu$^{161}$ are isofunctional to Glu$^{99}$ and Glu$^{172}$ in human GloI, respectively (supplemental Fig. 10). As a consequence, Glu$^{94}$ and Glu$^{345}$ are part of active site A between the N- and C-terminal domains, and Glu$^{272}$ and Glu$^{161}$ form active site B between the intermediate domains (Fig. 1B and supplemental Fig. 10).

At saturating substrate concentrations, both active sites have a similar catalytic turnover as reflected by similar $k_{cat1}^{app}$ values. Interpreting $K_{m1}^{app}$ and $K_{m2}^{app}$ as a measure of substrate affinity, we suggest that active site A, including Glu$^{94}$ and Glu$^{345}$, is a high-affinity binding site for the diastereomers. This reaction center is half-saturated in the lower micromolar concentration range, whereas active site B (formed by Glu$^{272}$ and Glu$^{161}$) is a low-affinity binding site that is half-saturated in the higher micromolar concentration range. On the one hand, these differences might facilitate the development of relatively specific glyoxalase inhibitors as novel antimalarial drugs. On the other hand, such specific inhibitors will probably inactivate only one of the two active sites. Provided that methylglyoxal is the physiologically most relevant substrate of PfGloI, it is more promising to target the high-affinity binding site because of its significantly higher catalytic efficiency. However, the range of physiological substrates and their stage-dependent concentrations, functions, and toxicities in *P. falciparum* are not known (see below).

The pH Optima of Both Reaction Centers Are Similar—PfGloI has a very broad pH optimum with two small local maxima at pH 7.0 and 7.5 and a third local maximum at pH ~5.8. We wanted to know whether both active sites have similar pH profiles or different overlapping pH optima and therefore compared the pH dependence of the enzymatic activities of wild-type PfGloI and the glutamate mutants (Fig. 4). The activity of all enzymes decreased significantly above pH 8.0 and showed a very similar insensitivity to pH changes between 6.5 and 8.0. In addition, the functional active sites of PfGloI(E91Q) and PfGloI(E345Q) were sensitive to pH values <6.5. Because both active sites of the wild-type enzyme were not strikingly inactivated at pH values ranging from 5.5 to 6.5, the sensitivity of PfGloI(E91Q) and PfGloI(E345Q) seems to be due to rapid inacti-
v = V_
+ V_ = k_at(E)[S] + k_0[E][S] \tag{1}

Values obtained from the fit (Fig. 5) qualitatively confirmed the theory of a high- and low-affinity binding site; however, \( k_{mb} \) is too large to explain the activity of \( Pf\text{GloI}(E91Q) \), \( Pf\text{GloI}(E345Q) \), and \( Pf\text{GloI}(E91Q/E345Q) \) at lower substrate concentrations (Fig. 3). Calculated S.D. values of \( k_{int} \) despite the relatively accurate measurements, also suggest that Equation 1 does not perfectly explain the kinetics of \( Pf\text{GloI} \). Other, even more important evidence contradicts the hypothesis that two independent active sites are sufficient to explain the biphasic kinetics: inactivation of one of the two reaction centers does not result in monophasic kinetics of the mutants. One might argue that the glutamate replacements are not sufficient and that a residual activity of the modified active site causes the biphasic kinetics of the six active mutants. Indeed, this cannot be absolutely excluded for \( Pf\text{GloI}(E91Q) \) and \( Pf\text{GloI}(E272Q) \) because they might be slightly active with the S-diastereomer (see above). The hypothesis that glutamate replacements are not sufficient to inactivate \( Pf\text{GloI} \) in general, however, is disproved by the almost complete inactivation of \( Pf\text{GloI}(E161Q/E345Q) \). In addition, similar studies on active-site mutants of Glol from human (21) and yeast (13) showed that replacements of the conserved glutamate residues with glutamine are sufficient to decrease activity by several orders of magnitude. We therefore conclude that the modified reaction centers of at least \( Pf\text{GloI}(E161Q) \), \( Pf\text{GloI}(E161Q/E272Q) \), \( Pf\text{GloI}(E345Q) \), and \( Pf\text{GloI}(E91Q/E345Q) \) are inactivated. Thus, a residual turnover at the modified active sites cannot be the reason for the observed biphasic kinetics of all functional \( Pf\text{GloI} \) mutants.

Biphasic kinetics and a Hill coefficient < 1 are indicative of enzymes with negative subunit cooperativity (27, 28). Such enzymes have to be able to oligomerize and to adopt at least two different conformations. We analyzed \( Pf\text{GloI} \) by gel filtration chromatography and exclusively detected monomeric protein (data not shown), suggesting that there is no subunit cooperativity. Allosteric regulation is also possible for monomeric proteins in case they occur with different conformations and have more than one binding site. What is known about conformational changes in Glol from different organisms? NMR studies on yeast Glol showed a very low incorporation of solvent protons into product (29), suggesting that proton transfers during catalysis occur at a highly protected active site. Fluorescence quenching experiments (30, 31) and proteolytic susceptibility studies (31) revealed that human Glol adopts different conformations in a substrate-dependent manner. A comparative analysis of the crystal structures of human Glol suggested that, during catalysis, several side chains at the active site adopt alternative positions and that a flexible loop is closing over the active site (11). Conformational changes during catalysis are also in agreement with the observed phosphate-dependent inactivation of our \( Pf\text{GloI} \) glutamate mutants (see above). Furthermore, wild-type \( Pf\text{GloI} \) and \( Pf\text{GloI}(E161Q/E345Q) \) were treated with subtilisin in the absence or presence of S-hexylglutathione or substrate, resulting in different time-dependent degradation patterns (Fig. 6). Binding of S-hexylglutathione slowed the degradation of the wild-type enzyme and \( Pf\text{GloI}(E161Q/E345Q) \) in comparison with uncomplexed protein. The time-dependent degradation patterns of \( Pf\text{GloI} \) and \( Pf\text{GloI}(E161Q/E345Q) \) in the presence of substrate were similar to samples with uncomplexed protein as far as the intensities of the ~45-kDa band are concerned, whereas the intensities of three bands at ~30 kDa seemed to depend on whether active enzyme or the almost completely inactive mutant was used. A plausible interpretation of our proteolytic susceptibility analy-

### TABLE 2

| Enzyme                  | \( k_{cat}^{app} \) | \( k_{cat}^{app} \) | \( k_{cat}^{app} \) | \( k_{cat}^{app} \) | \( k_{cat}^{app} \) |
|------------------------|--------------------|--------------------|--------------------|--------------------|--------------------|
| Wild-type              | 178 ± 30 (100%)    | 285 ± 12 (100%)    | 16 ± 3 (100%)      | 103 ± 21 (100%)    | 20.8 ± 2.9 (100%) |
| E91Q                   | 99 ± 25 (56%)      | 153 ± 33 (54%)     | 88 ± 7 (550%)      | 204 ± 4 (198%)     | 1.74 ± 0.37 (8%)  |
| E345Q                  | 80 ± 3 (45%)       | 115 ± 8 (40%)      | 88 ± 7 (550%)      | 191 ± 11 (185%)    | 1.33 ± 0.18 (6%)  |
| E91Q/E345Q             | 75 ± 2 (42%)       | 115 ± 12 (40%)     | 88 ± 4 (500%)      | 192 ± 2 (186%)     | 1.43 ± 0.09 (7%)  |
| E272Q                  | 138 ± 14 (78%)     | 151 ± 20 (53%)     | 12 ± 1 (75%)       | 12 ± 1 (75%)       | 12.5 ± 2.7 (60%)  |
| E91Q/E272Q             | 121 ± 24 (68%)     | 138 ± 23 (48%)     | 11 ± 1 (69%)       | 12.5 ± 2.7 (60%)   | 0.75 ± 0.06       |
| E161Q                  | 220 ± 20 (56%)     | 151 ± 30 (40%)     | 9 ± 1 (56%)        | 21 ± 8 (20%)       | 12.6 ± 4.5 (61%)  |
| E161Q/E272Q            | ND                 | ND                 | ND                 | ND                 | ND                 |
| E161Q/E345Q            | ND                 | ND                 | ND                 | ND                 | ND                 |

**FIGURE 3.** The steady-state kinetics of wild-type \( Pf\text{GloI} \) and glutamate mutants are biphasic. All assays were performed with enzymes that were purified in MOPS buffers. The formation of S-1-lactoylglutathione at 30 °C was followed spectrophotometrically at 240 nm in buffer containing 50 mM MOPS/NaOH (pH 7.0). Results from representative single experiments are shown as Edie-Hofstee (left panels), Hanes (middle panels), and Hill (right panels) plots (28). Circles represent data points at lower and higher substrate concentrations, respectively; squares represent data points that were not used for linear regression analysis. \( Pf\text{GloI}(E91Q)/Pf\text{GloI}(E345Q)/Pf\text{GloI}(E91Q/E345Q) \) and \( Pf\text{GloI}(E272Q)/Pf\text{GloI}(E161Q)/Pf\text{GloI}(E161Q/E272Q) \) each have very similar kinetics and form two distinguishable groups. WT, wild-type \( Pf\text{GloI} \).
sis is that PfGloI adopts at least two different conformations and that the distribution between those conformations depends on ligand binding. Even if both active sites of monomeric GloI are able to adopt different conformations, this is not sufficient to explain the biphasic kinetics; for this, the conformational changes have to be triggered in a concentration-dependent manner. Do the inactivated reaction centers still bind the substrate? This is probably the case because PfGloI(E161Q/E345Q) (with no functional active site) can be purified by an S-hexylglutathion-agarose column, and the proteolytic susceptibility of PfGloI(E161Q/E345Q) also depends on the absence or presence of ligand or substrate binding.

Proteolytic susceptibility assays of PfGloI with subtilisin were performed at 25 °C in the presence or absence of either 20 mM S-hexylglutathione as a stable ligand (L) or 5 mM hemithioacetal as a substrate (S). Samples were withdrawn from the assay mixture at the indicated time points, and the reaction was stopped before analysis by reducing SDS-PAGE. Equal sample volumes were loaded onto each lane. A, wild-type PfGloI (~15 μM) treated with 200 milliunits/ml subtilisin; B, PfGloI(E161Q/E345Q) (~10 μM) treated with ~50 milliunits/ml subtilisin.
The apparent steady-state kinetic constants of PfGloI were determined from Hanes plots (for example, see Fig. 7). Similar constants were obtained from Lineweaver-Burk and Eadie-Hofstee plots. All values given were averaged from two independent transformation/expression/purification experiments.

| Enzyme          | $k_{cat1}$ | $k_{cat2}$ | $K_{m1}$ | $K_{m2}$ | $k_{cat1} / k_{cat2}$ | $n_{H1}$ | $n_{H2}$ |
|-----------------|------------|------------|----------|----------|-----------------------|----------|----------|
| Wild-type       | 178 ± 30  | 285 ± 12  | 16 ± 3   | 103 ± 21 | 20.8 ± 2.9 (100%)     | 0.56 ± 0.02 | 1.01 ± 0.01 |
| R22E            | 75 (42%)  | 280 (98%) | 32 (200%)| 445 (432%)| 8.8 (42%)             | 0.7      | 1.0      |
| PfGloI(R22E/E91Q/E345Q) | 38 (21%)  | 86 (538%) | 0.4 (2%) |          |                      | 1.0      | 1.0      |

FIGURE 7. Alteration of the potential glutathione-binding site results in decreased substrate affinity of PfGloI(R22E) and monophasic kinetics of PfGloI(R22E/E91Q/E345Q). All assays were performed with enzymes that were purified in MOPS buffers. The formation of S-d-lactoylglutathione at 30 °C was followed spectrophotometrically at 240 nm in buffer containing 50 mM MOPS/NaOH (pH 7.0). Measurements were averaged from two independent transformation/expression/purification experiments, and the data are shown in Michaelis-Menten, Eadie-Hofstee, Hanes, and Hill plots (from left to right) (28). The $k_{cat}$, $k_{cat1}$, $k_{cat2}$, $k_{cat1}/k_{cat2}$, and $n_{H1}$, $n_{H2}$ values for both mutants are listed in Table 3. Furthermore, the Michaelis-Menten plot of PfGloI(R22E) was fitted using Equation 1. The values obtained for $k_{cat1}$ and $k_{cat2}$ are 17 and 455 s⁻¹, respectively. The values obtained for $K_{m1}$ and $K_{m2}$ are 60 and 670 μM, respectively.

TABLE 3

Mutation of Arg²² affects substrate binding

The apparent steady-state kinetic constants of PfGloI were determined from Hanes plots (for example, see Fig. 7). Similar constants were obtained from Lineweaver-Burk and Eadie-Hofstee plots. All values given were averaged from two independent transformation/expression/purification experiments.
are able to adopt two discrete conformations and are allosterically coupled.

Model of Positive Homotropic Allosteric Coupling in PfGloI Mutants—On the basis of our findings that PfGloI adopts different conformations and that the biphasic kinetics of active-site mutants are influenced by binding of the substrate to the modified active site, we suggest the reaction scheme shown in Fig. 8A. Equation 2 is derived from this scheme.

\[
V = \frac{k_3 K_s K_m [E][S]}{K_i K_m + (K_K e + K_6)[S] + (K_6 + 1)[S]^2}
\]

(Eq. 2)

when it was not clear whether glyoxalases catalyze a one- or two-substrate reaction, Mannervik et al. observed biphasic kinetics for yeast GloI that are very similar to the kinetics of PfGloI (see Eadie–Hofstee plot in Fig. 1 of Ref. 32). The authors’ interpretations are not in accordance with later models favoring the one-substrate reaction (Fig. 1A), and the cause of the biphasic kinetics remains unclear. We think that two different active sites of monomeric yeast GloI are plausibly related to the Michaelis-Menten equation. This might be due to the similar kinetic values for both active sites (resulting in very similar denominators in Equation 1). However, in 1974, during a time when it was not clear whether glyoxalases catalyze a one- or two-substrate reaction, Mannervik et al. observed biphasic kinetics for yeast GloI that are very similar to the kinetics of PfGloI (see Eadie–Hofstee plot in Fig. 1 of Ref. 32). The authors’ interpretations are not in accordance with later models favoring the one-substrate reaction (Fig. 1A), and the cause of the biphasic kinetics remained unclear. We think that two different active sites of monomeric yeast GloI are plausibly related to the Michaelis-Menten equation. This might be due to the similar kinetic values for both active sites (resulting in very similar denominators in Equation 1). However, in 1974, during a time
coupling between both active sites of monomeric GloI might be the correct explanation for this observation. We therefore suggest a re-evaluation of old data on yeast GloI and a reinvestigation of yeast active-site mutants.

Does homodimeric GloI also possess biphasic kinetics and an allosteric coupling? So far, there are no data supporting this hypothesis, although this might theoretically be the case provided that both identical active sites adopt different conformations in a concentration-dependent manner. Further studies are required to answer this question.

Speculations on the Physiological Functions of Monomeric GloI—Is the structural variability of GloI linked to special functional or regulatory features in vivo? The glyoxalase system has been known for almost a century since its discovery in 1913 (33). To date, we know a lot about the structural variability of glyoxalases, whereas our understanding of the physiological functions is far from complete. Studies on GloI from different organisms support the theory that one main function of the glyoxalase system is the detoxification of methylglyoxal formed, for example, during glycolysis (3). In this regard, positive homotropic allosteric coupling of PfGloI might be especially advantageous to maintain homeostasis because, at very low substrate concentrations, the enzyme has a higher affinity, decreasing the concentration of free 2-oxoaldehydes, whereas at high substrate concentrations, a conformational change leads to the more rapid turnover of the substrate. Indeed, malaria parasites have to adapt to a wide variety of different environments and glucose concentrations (for example, intracellular developmental stages in liver cells or erythrocytes and extracellular stages in human blood or the midgut of the mosquito). The situation is in some ways comparable with yeast and other Ascomycetes because they have to cope with environmental glucose concentrations that can temporally change over several orders of magnitude as well. Shifts in glycolytic fluxes might therefore be an important element that favors the development of allosteric coupling of (monomeric) GloI.

Both $K_m^{\text{MP}}$ values for PfGloI (Fig. 8C) are much smaller than those for yeast GloI (0.30 and 0.24 $\mu$M) (13), resulting in a 5-fold higher catalytic efficiency despite slightly smaller $k_{\text{cat}}$ values. These differences in $k_{\text{cat}}$ and $K_m$ values might reflect different physiological substrate concentrations. Another possibility is that both enzymes are optimized for different substrates. So far, the stage-dependent intracellular concentration of methylglyoxal and even the existence of different 2-oxoaldehydes in $P. falciparum$ are unclear. In yeast, the addition of external methylglyoxal under different growth conditions (34, 35) but also the kinetics (36, 37) and the effects (38) of the glycolysis-dependent internal formation of methylglyoxal were studied. Despite such great advances in understanding the metabolism of methylglyoxal, it has to be kept in mind that, depending on the type of organism investigated, the physiological sources of 2-oxoaldehydes can differ (for review, see Ref. 3), and novel sources, as well as the existence of alternative 2-oxoaldehydes, cannot be ruled out for $P. falciparum$ and other eukaryotes. The activity of GloI from yeast is quite insensitive to structural variations of the 2-oxoaldehyde (1, 39), and it was therefore hypothesized that glyoxalases in general have a very broad substrate spectrum. Yet, we assume that this is not necessarily the case because the broad substrate spectrum of yeast GloI might be due to the two different active sites reflecting a metabolic adaptation to different classes of 2-oxoaldehydes. For example, a significantly lower catalytic efficiency with a certain substrate might be compensated by monomeric GloI having a second, structurally different active site. Because GloI from yeast and $P. falciparum$ is monomeric, it would be worth comparing the catalytic efficiencies of all active sites with different substrates to confirm or disprove these hypotheses.

Although it is accepted that methylglyoxal leads to harmful advanced glycation end products (see Ref. 40 for review), which certainly play a role in diseases such as diabetes (41), very recent findings showed that methylglyoxal also causes protein modifications that can lead to signal transduction and regulation of gene expression (42). Thus, it is possible that allosteric regulation of PfGloI might play a role in the conversion of 2-oxoaldehydes as signal molecules. Another possibility is that the conformational changes in PfGloI may directly influence signal transduction pathways.

Conclusion—Previous studies on GloI from human (21), yeast (13), and other organisms were usually performed in phosphate buffers. We would like to recommend the use of MOPS buffers for future analyses (especially of active-site mutants) because of possible negative and disturbing effects due to an interaction between phosphate and the metal ion at the active site. We have shown that PfGloI has two functional active sites with similar catalytic activities and pH profiles but different substrate affinities. A plausible model of our kinetic data suggests that the hemithioacetal substrate acts as a positive homotropic allosteric regulator of the monomeric enzyme. Substrate binding to the first reaction center induces or stabilizes a different conformation of the second active site. As a consequence, $k_{\text{cat}}$ is increased, and substrate affinity is lowered in comparison with the conformation in the absence of the allosteric regulator. Similar and potentially physiologically relevant effects for monomeric (and maybe dimeric) GloI from other organisms could have been overlooked and should be considered in future studies.

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