Identification of Acid-Base Catalytic Residues of High-Mr
Thioredoxin Reductase from Plasmodium falciparum*‡§

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High-Mr, thioredoxin reductase from the malaria parasite Plasmodium falciparum (PfTrxR) contains three redox active centers (FAD, Cys-88/Cys-93, and Cys-535/Cys-540) that are in redox communication. The catalytic mechanism of PfTrxR, which involves dithiol-disulfide interchanges requiring acid-base catalysis, was studied by steady-state kinetics, spectral analyses of anaerobic static titrations, and rapid kinetics analysis of wild-type enzyme and variants involving the His-509-Glu-514 dyad as the presumed acid-base catalyst. The dyad is conserved in all members of the enzyme family. Substitution of His-509 with glutamine and Glu-514 with alanine led to TrxR with only 0.5 and 7% of wild type activity, respectively, thus demonstrating the crucial roles of these residues for enzymatic activity. The H509Q variant had rate constants in both the reductive and oxidative half-reactions that were dramatically less than those of wild-type enzyme, and no thiolate-ribonucleotide reductase, methionine oxide reductase, 2-Cys peroxiredoxins, and a number of transcription factors (1–4). Thus, the function of the thioredoxin system is crucial for cell proliferation, protection against reactive oxygen species, and signal transduction. Levels and activities of the thioredoxin system were found to be increased by at least an order of magnitude in many tumor cell lines (1), indicating that TrxR is critical to their viability, possibly by enhancing both the antioxidant capacity of the cell and the production of nucleotides. The importance of TrxR to cancer cells suggests that it would be an attractive chemotherapeutic target (5–8).

Tumor cells and malarial parasites have many common features, including high metabolic rates and rapid cell division. Approximately 500 million cases of malaria caused by infection with the protozoan parasite Plasmodium are reported annually, resulting in up to 3 million deaths, 75% of them being African children. Malaria not only has major health implications, but also causes substantial economic losses running into the billions of dollars in endemic areas. Because of increasing resistances of the parasites against antimalarial drugs, new and better chemotherapies are urgently required (9, 10). Plasmodium lacks both glutathione peroxidase and catalase, indicating that the thioredoxin system is particularly important for multiple roles, including reduction of glutathione, protection against oxidative stress, and biosynthesis of thymine. Indeed, TrxR has already been genetically validated in Plasmodium as a drug target (11). A first screen of a Pfizer chemical library identified nitrophenyl compounds as potential leads for development of clinical TrxR inhibitors (12). To date three compounds showing antimalarial activity as well as specific inhibition of PfTrxR over human TrxR are available (13, 14). To facilitate these investigations, a colorimetric microtiter assay has been developed that is suitable for high throughput inhibitor screening on PfTrxR (14).

TrxR occurs in both high and low molecular weight forms in Nature. The low-Mr TrxR class comprises enzymes found in Escherichia coli, fungi, plants, and the protozoan parasite, Trichomonas vaginalis (15–18). High-Mr, TrxR is present in mammals, insects, worms, and the malaria parasite, Plasmodium falciparum (5, 6, 15, 19, 20). The two classes are distinguished by molecular size, by the number of redox active centers, and by the mechanism by which they transfer reducing equivalents from the apolar part of the active center to the protein surface (15, 21).

High-Mr, TrxRs have many similarities to glutathione reductase and other disulfide oxidoreductases, but they are more complex (22). These homodimeric proteins contain three
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redox-active centers that are in redox communication: a tightly bound FAD, a pair of redox-active cysteines on one of the subunits that we will refer to as the N-terminal pair (Cys-88 and Cys-93 in Scheme 1, for PfTrxR), and a pair of redox-active cysteines or a cysteine-selenocysteine pair that we will refer to as the C-terminal pair (Cys-535/His-536 and Cys-540/His-536, for PfTrxR) located on the second subunit (15, 22, 23, 24). The reductive and oxidative half-reactions of Drosophila melanogaster TrxR (DmTrxR) have been examined in some detail to obtain a better understanding of the underlying catalytic mechanism of high-Mr TrxRs. Scheme 1, which outlines the various intermediates and redox reactions, resulted from these studies (25).

Catalysis, reducing equivalents are transferred through the active site redox centers of the homodimeric protein to effect reduction of thioredoxin disulfide (Scheme 1). NADPH reduces the FAD on the re side of the isalloxazine ring to form the FADH⁻ to NAPDH charge-transfer complex (CTC). The reduced flavin is reoxidized by the N-terminal disulfide on the si side of the flavin ring to form a thiolate-flavin charge-transfer complex between Cys-93 (flavin-interacting thiol) and the FAD. The nascent interchange thiol (Cys-88) reacts with the C-terminal disulfide (Cys-535-Cys-540) in a dithiol-disulfide interchange reaction, and finally, the C-terminal dithiol reduces Trx (a 13-kDa protein) in another dithiol-disulfide interchange. These intermediates can be observed by analyses of spectral changes at diagnostic wavelengths (see Table 1). From this description and Scheme 1, it can be seen that catalysis involves cycling between the EH₂ and EH₄ redox states. The reduction of Eox by NADPH is a priming reaction.

Although the overall catalytic mechanisms of all high-Mr TrxR have been shown to be similar (15, 22, 23, 25, 27), the enzymes show differences in the composition of their C-terminal redox-active sites. Mammalian and Caenorhabditis elegans TrxR contain a cysteine adjacent to a selenocysteine residue, whereas insect TrxR contains two adjacent cysteine residues, and PfTrxR has C-terminal redox-active cysteines that are separated by four amino acids (5, 6, 15, 25, 27) (supplemental Fig. S1). Dithiol-disulfide interchange reactions, such as that between the N-terminal dithiol and the C-terminal disulfide, require the assistance of an acid-base catalyst, as shown for disulfide oxidoreductases such as glutathione reductase. Structural studies of human glutathione reductase revealed that two amino acid residues, histidine and glutamic acid, are juxtaposed to the N-terminal disulfide; these residues are present in a highly conserved HPXXXE motif. The NE2 of the imidazole ring of this histidine is near the interchange-thiol of the N-terminal pair, while the epsilon carboxyl of glutamic acid appears to be hydrogen bonded to ND1 of this same histidine (28–30). Therefore, this His-Glu dyad is ideally positioned to promote dithiol-disulfide interchange between the active site cysteines and glutathione during catalysis by glutathione reductase. In addition to the structural results, rapid kinetics analyses of glutathione reductase and the highly related dihydrolipoamide dehydrogenase showed that an acid-base catalyst is necessary for full catalytic activity (31–33).

The recent crystal structures of mouse mitochondrial TrxR in the oxidized and reduced states (34) reveal that it also has a His-Glu dyad, similar to that in glutathione reductase, and it is well positioned to catalyze dithiol-disulfide interchange between the N-terminal and C-terminal dithiol pairs. In TrxR from P. falciparum, which has considerable sequence homology to mouse TrxR, His-509 and Glu-514 comprise the analogous His-Glu dyad. The properties of site-directed variants of the residue analogous to His-509 have been studied in most of the members of this enzyme family. However, the conserved glutamate has only been experimentally examined previously in lipoamide dehydrogenase and glutathione reductase (31–33).
TABLE 1
Spectral properties of selected *P. falciparum* TrxR enzyme species

The spectral features listed here were utilized for characterization of PfTrxR wild-type and variant enzyme species.

| Enzyme species$^a$ | Characteristic wavelength (nm) | $\varepsilon$ at the characteristic wavelength ($\text{mM}^{-1} \text{cm}^{-1}$) | $\varepsilon$ at distinct wavelengths ($\text{mM}^{-1} \text{cm}^{-1}$) |
|-------------------|-------------------------------|---------------------------------|---------------------------------|
| $E_{ox}$          | 462                           | 11.3                            | 8.7, 3.2, 1.2                   |
| $E_{red}$         | 462                           | 0.76                            | 1.2                             |
| $E_{red}^{\text{NADPH}}$ | 670                           | 2.2–3.2                        | 3.2                             |
| $E_{red}^{\text{NADP}}$ | 540                           | 2.2                             | 0.2–0.6                         |
| $E_{red}^{\text{NADPH}}$ | 540                           | 1.13                            | 0.2                             |

$^a$ For nomenclature of the enzyme species, see text and Scheme 1.

$^b$ When enzyme-bound FAD is reduced to FADH$^-$, a large fraction of the visible absorbance is lost. Fig. 2A, curve 6, is a typical reduced enzyme FAD spectrum. This particular spectrum was generated by adding 3 eq of dithionite in the presence of ~1% methyl viologen (MV), which acted as a mediator to transfer electrons from the added dithionite to FAD. The small absorbance bump at 394 nm is caused by reduced MV.

$^c$ The increase in absorbance at this wavelength is transiently observed in stopped-flow kinetic and spectral traces, occurring from ~5 ms to 200 ms depending on whether wild-type or a variant species is being observed. Fig. 2A, curve 3 and Fig. 2B, curve 3 approximate partial formation of these complexes in equilibrium with oxidized enzyme.

$^d$ See Ref. 44 for further characterization of these enzyme-pyridine complexes.

The role of the glutamate in the acid-base dyad has also been studied by molecular dynamics and density functional calculations, which predict its importance in making histidine a better catalyst (26).

The role of acid-base catalysis in the mechanism of high-M$_r$ TrxRs has not been completely addressed. Therefore this study focuses on acid-base catalysis in the reaction cycle of the high-M$_r$ TrxR from the malaria parasite, *P. falciparum*. Knowledge of the precise roles of individual active site residues of the *Plasmodium* enzyme will increase our understanding of differences in the catalytic mechanisms between parasite and mammalian TrxR. This knowledge will, together with the known structural differences, be useful for the design of inhibitors that selectively act against the *Plasmodium* protein (see below).

**EXPERIMENTAL PROCEDURES**

**Materials**—Recombinant *P. falciparum* TrxR (PfTrxR) wild-type and PfTrxR variant proteins were generated as described in (19), and PfTrxR was recombinantly expressed and purified as described in (35, 36). Ni-NTA agarose was purchased from Qiagen. NADPH was from Melford and 5,5$’$-dithiobis (2-nitrobenzoic acid) (DTNB), methyl viologen, sodium hydrosulphite (sodium dithionite), and sodium borohydride were from Sigma.

**Site-directed Mutagenesis—** *P. falciparum* TrxR wild-type cloned in pJC40 (19) was used as a template for generating the site-directed mutant PfTrxR E514A. The oligonucleotides used are shown in supplemental Table S1. The mutagenesis was performed according to Refs. 19 and 37. All other mutant proteins were previously generated as described in Ref. 19.

**Recombinant Expression and Purification of TrxR Wild-type and Variant Proteins**—After the sequences of all PfTrxR expression constructs were confirmed by automated sequencing (The Sequencing Service, University of Dundee), they were transformed into *E. coli* BLR (DE3), and expression of recombinant proteins was performed as previously described (19). The proteins were purified in a batch procedure using Ni-NTA agarose according to the manufacturer’s recommendations (Qiagen). PfTrxR concentrations were determined using the molar extinction coefficient at 462 nm of 11,300 M$^{-1}$ cm$^{-1}$ (19).

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PfTrxR concentrations were determined by using the molar extinction coefficient at 280 nm of 11,700 M$^{-1}$ cm$^{-1}$ (36) or the Bradford method (38). The purity of all proteins was analyzed by SDS-PAGE before the PfTrxR proteins were precipitated with 80% ammonium sulfate for long term storage at 4 °C. Before use, the proteins were dialyzed extensively against 50 mM potassium phosphate buffer pH 7.6 containing 1 mM EDTA. PfTrxR was isolated in the oxidized state, in contrast to *Dm*TrxR that was partially reduced when isolated and required oxidation with ferricyanide prior to use (25). The integrity of the variant proteins was verified by circular dichroism analysis (supplemental Fig. S2).

**Steady-state Kinetic Analyses**—The steady-state kinetic parameters of PfTrxR with NADPH and thioredoxin or DTNB were analyzed by two different spectrophotometric assay systems: (i) DTNB-reduction was measured in an assay mixture containing 50 mM potassium phosphate buffer pH 7.6, 1 mM EDTA, with varying concentrations of NADPH (0.5–200 $\mu$M), 1–20 $\mu$g of TrxR (wild-type or variants) to determine the $K_m$ for NADPH. The increase of absorbance at 412 nm was followed spectrophotometrically (Shimadzu UVPC 2501), and the specific activities of PfTrxR wild-type or variants were determined using the molar extinction coefficient of TNB$^-$ released during the reaction (13,600 M$^{-1}$ cm$^{-1}$ per TNB$^-$ released) (39). (ii) The reduction of thioredoxin was assayed according to Lutheran and Holmgren (40) in 50 mM potassium phosphate, pH 7.6, 1 mM EDTA, 0.2 mg ml$^{-1}$ insulin, 0.5 to 20 $\mu$g of TrxR (wild-type and variants) and varying concentrations of Trx (2–50 $\mu$M) at 200 $\mu$M NADPH to determine the $K_m$ values for Trx. The change in absorbance because of the oxidation of NADPH was followed spectrophotometrically at 340 nm, and the specific activities were calculated using the extinction coefficient of 6,220 M$^{-1}$ cm$^{-1}$.

**Static Anaerobic Titrations with NADPH or Dithionite**—Wild-type or variant PfTrxR (10–15 $\mu$M in 1 ml), freshly dialyzed (against 50 mM potassium phosphate containing 1 mM EDTA, pH 7.6) was transferred into an anaerobic cuvette and made anaerobic using ~10 cycles of argon/vacuum before 2-$\mu$L
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additions of anaerobic NADPH (1.5 mM) were titrated into the protein solution as described previously (23). After each addition of NADPH, spectra of the proteins between 350 nm and 700 nm were recorded in a Cary 300 spectrophotometer (Varian).

The proteins were also anaerobically reduced with sodium dithionite (41). Sodium dithionite was dissolved in anaerobic 50 mM pyrophosphate, pH 9.0 to give a final concentration of 1.5 mM. Additions of this stock solution (5 μM) were titrated into the anaerobic wild-type PfTrxR or variant proteins and spectra between 350 nm and 700 nm were recorded in a Cary 300 spectrophotometer.

Reductive Half-reactions, Stopped-flow Rapid Kinetics—The reactions of PfTrxR wild-type and variants with NADPH at pH 7.6 were recorded using a SF-61 DX2 double mixing stopped-flow system (Hi-Tech). Anaerobic wild-type or variant PfTrxR (30 μM) was mixed with 0.5 to 6 (for \(k_c\) calculations) equivalents of anaerobic NADPH at 5 °C, and the reductive half-reactions were followed using either the photodiode array mode, when changes of the complete spectra of the proteins were followed (between 300 nm and 700 nm), or the photomultiplier mode when changes at single diagnostic wavelengths (355, 440, 540, and 670 nm, see Table 1) were analyzed.

Effect of pH on the Reductive Half-reaction of Wild-type and H509Q PfTrxR—The effect of increasing pH on the reductive half-reaction was analyzed by dialyzing the protein into a buffer containing 50 mM potassium phosphate and 1 mM EDTA at pH 7.0, 7.6, and 8.2 before reacting it with 0.5–12 equivalents of anaerobic NADPH in the stopped-flow spectrophotometer.

Oxidative Half-reactions, Stopped-flow Rapid Kinetics—Wild-type or variant PfTrxR (40 μM) were anaerobically reduced either with 2.2 equivalents of dithionite or 2.2 equivalents of NADPH to produce EH4. The reduced enzymes were then mixed with 3–20 equivalents of PfTrxR in the stopped-flow spectrophotometer and the reactions were followed until complete. Spectra were recorded at the end of the reaction.

All stopped-flow data were fit to multiple exponential functions for sequential reactions using Program A, written at the University of Michigan by Yong Chang, Jung-yen Chiu, Joel Dinverno, and David Ballou, as described in Ref. 25.

RESULTS

Catalytic Activity of PfTrxR Variants—The substitution of His-509 by glutamine had large effects on the catalytic activity of PfTrxR. The value of \(k_{cat}\) for the reduction of PfTrxR by H509Q PfTrxR was only 0.5% of that of wild-type PfTrxR, but the \(K_m\) was unchanged (Table 2). The pH dependence of the kinetic parameters \(k_{cat}\) and \(k_{cat}/K_m\) have been studied in TrxR from D. melanogaster, DmTrxR. Given the structural and mechanistic similarity between the enzymes from the two sources, it seems likely that similar pH dependence will be found to apply to PfTrxR.

The substitution of Glu-514 by alanine also affected the catalytic activity of PfTrxR, but not as strongly as in H509Q. The \(k_{cat}\) for the reduction of Trx by E514A was only 7.5% of that of wild-type PfTrxR, and the \(K_m\) was unchanged (Table 2). The effects of these two mutations on steady-state kinetic parameters indicate pivotal roles for both His-509 and Glu-514 in PfTrxR catalytic activity.

TABLE 2

| Protein | \(K_m\) \(\mu M\) | \(k_{cat}\) \(\text{min}^{-1}\) |
|---------|-----------------|------------------|
| WT      | 3.4 ± 0.3       | 1770 ± 50        |
| H509Q   | 3.3 ± 0.5       | 8 ± 0.3          |
| E514A   | 4.0 ± 0.5       | 133 ± 4.5        |

Values are means ± S.D. of at least three independent measurements.
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When comparing this spectrum to that of the NADPH-reduced protein, it is obvious that NADPH leads to the reduction of the flavin with formation of an FADH^-NADP^+ CTC, indicated by the very broad band from 525 to >700 nm without the formation of a clear thiolate-flavin CTC band. These results indicate that electron transfer from the reduced flavin to the N-terminal disulfide is inhibited in this variant. In H509Q the cysteinyl residue (Cys-93) is not deprotonated and does not charge transfer to the flavin, because of the absence of the stabilizing effect of the imidazolium positive charge of His-509. Thus, His-509 is critical to both the electron transfer functions and the stabilization of the CTC of PfTrxR.

Titration of E514A with either NADPH or dithionite gives a high yield of the thiolate-flavin CTC (ε_540 nm of 4.69 mM^-1 cm^-1 compared with 3.01 mM^-1 cm^-1 in wild-type enzyme using dithionite), and this is accompanied by a further shift to a shorter wavelength of the main flavin band from 462 nm to ~437 nm (Fig. 1C, dithionite-reduced). This result suggests that the exchange of the negative charge of glutamate by the apolar alanine enhances the CTC interaction between Cys-93 thiolate and the flavin. Thus, His-509 and Glu-514 appear to affect the formation and stability of different intermediates that occur during the reductive half-reaction of PfTrxR (Scheme 1), favoring EH_2(B) and EH_4(B).

Stopped-flow Studies of the Reductive Half-reaction of PfTrxR

Wild Type and Variants—Wild-type PfTrxR displays three observable kinetic phases during the reductive half-reaction with NADPH (Table 3, k_1 obs, k_2 obs, and k_3 obs). The mechanism of Scheme 1, which is based on previous observations with the less complex family members (32, 42), has at least eight reversible kinetic events for the reductive half-reaction, and even this is a minimal working model. During the first phase of the reductive half-reaction, the FADH^-NADP^+ CTC (EH_2(A) in complex with NADP^+, Scheme 1 and Table 1) is rapidly formed, as shown by the decrease in absorbance at 440 nm and the increase in absorbance at 670 nm (Fig. 2A, spectrum 3 and inset, traces 1 and 3). The first phase (between ~1 and 8 ms) is dependent on the concentration of NADPH and approaches a saturating rate of 700–800 s^-1 (Table 3). The first phase at 440 nm also corresponds to the first phase observed at 355 nm, which is caused by the oxidation of NADPH (Fig. 2A, inset, curve 4). This wavelength is the isosbestic point for oxidized enzyme, EH_2, and EH_4. The kinetic phases following the first phase are complex when more than 2 equivalents of NADPH are reacted. During the second phase of the reaction (between ~7 and ~50 ms), reducing equivalents are transferred from the reduced flavin to the N-terminal disulfide, resulting in formation of the thiolate-flavin CTC between the thiolate of Cys-93 and the FAD to form EH_2(B); this reaction, with an apparent rate constant of 360 s^-1 at the pH optimum of 7.6 (Table 3), is observed as increases in absorbance at both 440 nm and 540 nm (Fig. 2A, spectrum 5 and inset, curves 1 and 2). During this process, NADP^+ is displaced by NADPH, and this dissociation likely promotes reduction of the N-terminal disulfide by FADH^- . After dithiol-disulfide interchange reactions to form EH_3(D), a second equivalent of NADPH reduces TrxR to EH_4(A). We observe this process as a second phase of NADPH oxidation at 355 nm occurring at ~32 s^-1. However, the inset of
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TABLE 3

Rates determined for the reductive half reaction of wild-type and variants versus NADPH

The reactions were carried out in 50 mM potassium phosphate buffer, 1 mM EDTA, 5 °C, at the stated pH values.

| PfTrxR | pH | $k_1_{\text{obs max}}$ | $k_{2_{\text{obs}}}$ | Amp2/Amp2 + Amp3 | $k_3_{\text{obs}}$ | $K_{d_{\text{app}}} - \text{NADPH}^*$ |
|--------|----|------------------|------------------|-----------------|-----------------|-------------------------------|
| WT     | 7.0 | 720 (±134)        | 485$^b$          |                  | 20$^c$          | 14 (±3)                       |
| WT     | 7.6 | 793 (±104)        | 360$^b$          |                  | 20$^c$          | 25.3 (±2.5)                   |
| WT     | 8.2 | 815 (±10)         | 168 (±11)$^b$    |                 | 20$^c$          | 30 (±6)                       |
| H509Q  | 7.0 | 425 (±39)         | 10$^a$           | 0.08 (±0.02)     | 59 (±18)        |
| H509Q  | 7.6 | 567 (±72)         | 0.37 (±0.04)     | 0.07 (±0.04)     | 77 (±18)        |
| H509Q  | 8.2 | 663 (±75)         | 0.63 (±0.05)     |                  | 47 (±8)         |
| E514A  | 7.6 | 803 (±56)         | 11.2 (±1.5)      |                  |                 |
|        |     |                  |                  |                 |                 |

$^a K_{d_{\text{app}}} - \text{NADPH}$ values were determined from the hyperbolic increase in rate for the first kinetic phase, $k_{1_{\text{obs}}}$, as the [NADPH] increases. Thus, $k_{1_{\text{obs}}}$ reflects the rate of flavin reduction.

$^b$ This kinetic phase is only observed with more than 2 eq of NADPH and accounts for 25–45% of the total increase at $A_{440}$ nm for all pH values tested. The rate of flavin reoxidation is reflected by $k_{2_{\text{obs}}}$. The dependence of this rate (measured at 440 nm) on the concentration of NADPH (0.5 eq to >9 eq) appears to be hyperbolic. The rate decreases as the [NADPH] increases.

The rate constant $k_{3_{\text{obs}}}$ is complex, reflecting interchange between the N-terminal dithiol and the C-terminal disulfide and reaction with the second equivalent of NADPH.

$^c$ Not determined. At 2.96 eq of NADPH (the lowest amount tested) at a rate of 460 s$^{-1}$ was observed; therefore saturation had already been reached implying a very tight binding of NADPH at this pH. Thus, $k_{1_{\text{obs max}}}$ (425 s$^{-1}$) is an average of all concentrations used.

Fig. 2A shows that the absorbance at 440 nm continues to increase (at ~20 s$^{-1}$) after the decrease at 355 nm (32 s$^{-1}$) is complete (between ~50 and ~200 ms). We attribute this slower phase to EH$_4$(A) converting to EH$_6$(B). Thus, the absorbance increases seen at 440 nm caused by formation of FAD and at 540 nm (curve 2) signify nearly quantitative thiolate-flavin CTC.

It can be noted that in the reductive half-reaction with more than 2 equivalents of NADPH, fully reduced wild-type enzyme (EH$_4$) (Fig. 2A, spectrum 6) is not observed. This is consistent with Scheme 1, showing that catalysis occurs between EH$_2$ and EH$_4$.

We have attempted to quantify the two phases of NADPH oxidation observed at 355 nm; the change in extinction coefficient at 355 nm for NADPH oxidation is 4.85 mm$^{-1}$ cm$^{-1}$. However, the first reduced enzyme species formed, EH$_4$(A), is not isosbestic with the other EH$_3$ species and E$_{ox}$ at 355 nm; its extinction is 2.5 mm$^{-1}$ cm$^{-1}$ less than that of E$_{ox}$. Therefore, the total extinction change in the first phase to form FADH$^+$ and NADP$^+$ is ~7.3 mm$^{-1}$ cm$^{-1}$. We can calculate that the first phase corresponds to ~0.6 eq of NADPH oxidized. (We accounted for that part of the reaction that occurred in the dead time of the stopped-flow instrument.) The second phase at 355 nm corresponds to ~1.6 eq of NADPH oxidized.

The spectra recorded during the progress of the reductive half-reaction of PfTrxR H509Q (Fig. 2B) have a very different pattern when compared with those from the same reaction of wild-type enzyme (Fig. 2A). Although the rate for the formation of the FADH$^+$-NADP$^+$ CTC is about the same as that for the wild-type enzyme, the subsequent steps are several hundred-fold slower and result in a different distribution of intermediates from that of wild-type enzyme (Table 3). This demonstrates the important role for His-509 in the reactions subsequent to flavin reduction. In particular, the rapid formation and stabilization of the thiolate-flavin CTC in the wild-type enzyme (<300 ms; spectrum 5 in Fig. 2A) contrasts with the changes with the H509Q variant, where the spectrum of the FADH$^+$-NADP$^+$ CTC is stable for ~1 s (spectrum 3 in Fig. 2B).

The second phase (0.3–7 s) is characterized by an increase in absorbance at 440 nm and a decrease at 670 nm (Fig. 2B, inset, curves 1 and 3); this reflects transfer of reducing equivalents from the flavin to the N-terminal disulfide without formation of much thiolate-flavin CTC. The third phase (7-70 s), with increases in absorbance at 440 nm and 540 nm, may be caused by deprotonation of Cys-93 and stabilization of a small amount of thiolate-flavin CTC (Fig. 2B, spectrum 4 at 140 s) (see “Discussion”). These transitions occur in wild-type enzyme much more quickly and are complete by 100 ms.

Fig. 3 shows the changes in absorbance at 440 nm for reactions of H509Q with NADPH at pH 7.0, 7.6, and 8.2. The data were best fit using 3 exponential functions, and the observed rate constants are given in Table 3. The average value of $k_{2_{\text{obs}}}$ was 0.5 s$^{-1}$ at the two higher pH values; this step was caused by flavin reoxidation. In contrast, kinetic analysis of the data at pH 7.0, gave $k_{2_{\text{obs}}}$ of 5 s$^{-1}$, associated with a further small decrease at 440 nm, suggesting a different set of intermediates at the lower pH value. NADPH uptake (data not shown) was 0.6 equivalents in each of the first and second phases of the reaction at all pH values, indicating reduction beyond the EH$_4$ level. The data in Fig. 2B and Table 3 therefore demonstrate that His-509 is extremely important for facilitating the transfer of reducing equivalents from reduced flavin to the N-terminal disulfide as well as in the subsequent reactions.4,5

Fig. 2C shows spectra recorded during the reductive half-reaction of E514A at 1, 2, and 20 ms, and at 1.425 s. The inset shows the kinetics at 440, 540, and 670 nm. As observed in wild-type PfTrxR and in H509Q, the first phase of the reaction is essentially unchanged by the mutation (Fig. 2C, inset, curve 1; Table 3). The data show that the observed rate constants of the second and third phases of the reductive half-reaction, i.e. the

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5 Personal communication: H-H. Huang, L.D. Arscott, D.P. Ballou, and C.H. Williams, Jr. Study of the pH dependence of the reductive-half reaction in H464Q DmTrxR indicated that each of the three rates that comprise the overall reaction were favored by high pH, but the effect on $k_a$, reflecting transfer of reducing equivalents from FADH$^+$ to the N-terminal disulfide, and $k_b$, a complex rate constant reflecting interchange between the N-terminal dithiol and the C-terminal disulfide (as well as subsequent steps) was far greater than the effect on $k_c$, reflecting flavin reduction; at least one of the rates would be expected to be affected given the very low rate of the overall reaction in the variant. Determination of the pH dependence of the reductive half-reaction in wild-type DmTrxR is in progress; it can be seen at pH 8.0, that $k_a$ and $k_b$ are much more affected by the mutation than is $k_c$. These results add further evidence to the assignment of His-464 as the acid-base catalyst in DmTrxR. Study of the effect of pH on the oxidative half-reaction has just begun. These studies will be extended to PfTrxR and its variants.
formation and breakdown of the thiolate-flavin CTC, are slower relative to the wild-type enzyme at pH 7.6, $k_2$ by 30-fold and $k_3$ by 20-fold. This variant forms a slightly more intense thiolate-flavin charge-transfer band than does wild-type TrxR, as was seen in the static NADPH titration (Fig. 1C and spectrum 5 of Fig. 2C), and indicates that in the absence of Glu-514 efficient transfer of electrons through the active site of PfTrxR toward the C-terminal disulfide is restricted. This contrasts with the effects of removing His-509, where the amount and rate of formation of the thiolate-flavin CTC was greatly diminished.

Rapid Reaction Kinetic Analyses of the Oxidative Half-reaction of PfTrxR Wild-type and Variants—PfTrxR wild-type and variant proteins (pre-reduced to the EH4 level, with 2.2 equivalents of either dithionite or NADPH) were mixed with 0.5–6 equivalents of Pf thioredoxin disulfide (PfTrx) in the stopped-flow spectrophotometer. Changes in absorbance were recorded either at 490 nm where the largest changes were observed, or at 540 nm where disappearance of the thiolate-flavin CTC was best seen (Table 1). These data have been fitted to three exponentials (Table 4). Spectra recorded 16 min after initiating the reaction are shown in Fig. 4A for wild-type PfTrxR, pre-reduced with 2.2 equivalents of dithionite and reoxidized with a range of PfTrx concentrations; spectra of Eox and dithionite-reduced EH4 forms are shown for reference. Upon reaction of the dithionite-reduced wild-type protein with PfTrx, the enzyme rapidly (by 0.1 s) re-oxidizes to an equilibrium mixture of EH2 species, and much more slowly to Eox; the first phase of the reaction has a half-time of $t_{1/2}$ ~15 ms, whether observed at 490 nm or 540 nm (Fig. 4A, inset). There is no observable dependence of $k_1$ on the concentration of PfTrx, implying that PfTrx is fully bound very rapidly, and we are

150 ms; curve 5, 1.425 s. Inset, kinetic traces: curve 1, left y-axis, 440 nm; curve 2, right y-axis, 540 nm; and curve 3, right y-axis, 670 nm. (Rate constants are shown in Table 3.)
TABLE 4
Rates determined for the oxidative half-reaction of wild-type and variants reoxidized with PfTrx
Enzyme was pre-reduced at 5 °C, in 50 mM potassium phosphate buffer, 1 mM EDTA, pH 7.6, with either 2.0 eq of dithionite or 2.2 eq of NADPH to generate an EH1 species.

| Reductant           | PfTrxR | k1 obs (s^-1) | k2 obs (s^-1) | k3 obs (s^-1) |
|---------------------|--------|---------------|---------------|---------------|
| Dithionite-reduced  | WT     | 40 (±2)       | 0.16 (±0.14)  | 0.003 (±0.001) |
|                     | NADPH-reduced | 20–28       | 3.1 (±0.6)    | 0.05 (±0.03)  |
| NADPH-reduced       | H509Q  | 11.1 (±0.1)   | 0.0181 (±2e-4) | 1.3e-4 (±1e-5) |
|                     | E514A  | 10 (±2)       | 0.14 (±0.02)  | 0.004 (±0.003) |

Wild-type enzyme, pre-reduced with 2.2 equivalents NADPH, was reacted with excess PfTrx (Fig. 4B, inset, curves 1 and 2, and Table 4). The first phase, which was observed only at 540 nm as a small decrease in absorbance, occurred with a rate constant between 20 and 28 s^-1, and was most likely because of the dissociation of NADP+. Re-oxidation of the flavin was observed at 540 and 490 nm during the second phase, k2 obs at 3.1 s^-1. Note that the NADPH-reduced enzyme reacts with Trx more than 10-fold slower than does the dithionite-reduced enzyme (3.1 s^-1 versus 40 s^-1). The slower reaction is probably limited by the release of NADP+. The binding of NADP+ to the EH1 enzyme likely stabilizes the reduced flavin anion (EH1(A), Scheme 1).

The oxidative half-reactions of both the H509Q and E514A variants, reduced with 2.2 equivalents NADPH and subsequently reoxidized with 19–23 equivalents PfTrx were considerably slower than those of the wild-type protein; the progress of the reaction at 490 nm is compared with that for wild-type enzyme in the insets of Fig. 5, A and B. The half-times for the 2nd phase, (showing the large increase at 490 nm) representing reoxidation of H509Q or E514A PfTrx, were ~50 s or 6 s with apparent rate constants of 0.02 and 0.14 s^-1, respectively. Fig. 5A shows that the H509Q enzyme is almost fully reoxidized 16 min after mixing with ~19 equivalents PfTrx (Spectrum 3). The first phase observed in the kinetics of this variant, with a rate constant of 11 s^-1, represents only ~10% of the total extinction change at 490 nm (the rates are summarized in Table 4). This phase might be due in part, to the release of bound NADP+ rather than to re-oxidation. A similar kinetic trace was observed for E514A. The reoxidation of E514A enzyme appears to stop at a state in which the enzyme remains partially reduced (Fig. 5B, inset curve 2). It should be noted that the spectral characteristics of the reduced variants prior to reoxidation are not alike; the spectrum of H509Q (panel A) is like that of a mixture of oxidized and reduced flavin, while the spectrum of E514A (panel B) is typical of a thiolate-flavin charge-transfer complex. This confirms the findings of the reductive half-reaction experiments in which H509Q had virtually no capacity to stabilize the thiolate-flavin charge-transfer complex, whereas E514A stabilized even more of the complex than wild-type enzyme.

DISCUSSION
The major objective of this work has been to ascertain the roles of the putative acid-base catalytic residues in PfTrxR that facilitate the transfer of reducing equivalents from the flavin through the redox active cysteines to Trx, as shown in Scheme 1. A His-Glu pair is strictly conserved in this dithiol-disulfide flavin-dependent oxidoreductase family, and has been verified
The residues in both rat and mouse TrxRs analogous to His-509 and Glu-514 in PfTrxR are structurally near the N-terminal redox-active cysteines, as reviewed in the Introduction (34, 43). However, in high-Mr TrxRs, in addition to the initial reduction of the N-terminal Cys pair, acid-base catalysis is likely to also be required for dithiol-disulfide interchange from the N-terminal Cys pair to the C-terminal Cys- or Cys-Sec pair. In the rat enzyme a reasonably small movement of the C-terminal peptide is suggested to bring the selenolysulfide redox center close to the N-terminal cysteines and into proximity to the His-Glu pair, well positioned for catalysis of dithiol-disulfide interchange (43). The mobility of the C-terminal peptide, which permits these C-terminal residues to readily move between the apolar flavin region of the active site and the protein surface, is thought to be mechanistically important in that it allows the C-terminal selenolysulfide to interchange with the N-terminal dithiol in what we might call the “in” conformation, and to subsequently interchange with Trx in the “out” conformation (34, 43). A possible role in catalysis of the interchange between the N-terminal dithiol and the selenolysulfide by the His-Glu dyad is clear from the modeled structure of the C-terminal residues in the “in” conformation (34).

When considering this model for PfTrxR, there is one difference between the Plasmodium and the mammalian TrxR structures that must be mentioned as a caveat; there are four residues inserted between Cys-535’ and Cys-540’ in the parasite sequence that could modify the motions of the postulated swinging peptide. This structural and chemical difference is the basis for our suggestion that PfTrxR might be suitable for the design of inhibitors acting specifically against the Plasmodium protein. This study provides further knowledge about the catalytic and kinetic consequences that this difference in active site structure might have for the rational design of new inhibitors acting against the parasite enzyme. One difference might be that acid-base catalysis of the final interchange between the C-terminal selenolate-thiol and Trx might not be necessary in mammalian TrxR, given the likely low pKₐ of the selenolate at the protein surface.

Our results on the E514A variant show that with respect to wild-type enzyme, the kcat is >10-fold slower (Table 2), the reduction by NADPH to full formation of the thiolate-flavin CTC is >20-fold slower (Fig. 2, A and C and Table 3), and the reoxidation of EH₂ by PfTrxR is ~20-fold slower (Table 4). These results demonstrate the importance of Glu-514 in catalysis and are consistent with theoretical studies of this reaction (26). The molecular dynamics simulations and density functional theory ab initio calculations predict that a His-Glu dyad is a better catalyst of dithiol-disulfide interchange than is His alone, and that selenocysteine is a more favorable nucleophile than Cys, emphasizing again the catalytic differences between mammalian and parasite enzymes.⁶

Mutation of His-509 had a drastic effect on enzyme activity, consistent with the notion that this residue is the crucial base

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⁶ Four caveats should be noted about the theoretical studies. First, x-ray data for EH₂ was used to model the EH₂/EH₁ state; second, the structure used is of a Cys variant of the crucial selenocysteine (Sec) residue; third, thermo-dynamic calculations do not always reflect kinetic reality; and finally, the mechanisms in Schemes 3 and 4 of Ref. 24 use standard states with no allowance for the gradient of polarity within the active site. In spite of these reservations, the results are of interest.
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involved in catalysis of TrxR in a manner similar to the equivalent residue of other disulfide oxidoreductases (32). However, His-509 is able to function only minimally as the acid-base catalyst in the absence of Glu-514, i.e. in the E514A variant. These results suggest that the role of Glu-514 is to facilitate the acid-base catalysis by His-509, perhaps by stabilizing its positive charge and helping to orient the imidazolium side chain for optimal catalysis.

Anaerobic titrations of wild-type enzyme with NADPH or dithionite (Fig. 1) show that the thiolate-flavin CTC in which Cys-93 is the donor, is the dominant species in EH$_2$. Reduction of E$_{ox}$ to EH$_2$ is observed with 1 equivalent of NADPH, and the equilibrium between EH$_2$ and EH$_4$ lies far toward EH$_2$, as indicated by the high extinction coefficient of the CTC. As the second equivalent of NADPH is added, the spectral changes are subtle; the thiolate-flavin charge transfer is enhanced (~-34%) implying that EH$_4$ becomes the major species. Importantly, very little reduced flavin is observed. These changes are typical of those seen with other members of the enzyme family, e.g. glutathione reductase and lipoamide dehydrogenase (44).

In contrast to the results with wild-type enzyme, the H509Q variant protein showed almost no thiolate-flavin CTC in titrations with NADPH or dithionite. The major species appears to be the FADH$^-$-NADP$^+$ CTC, suggesting that His-509 not only stabilizes the thiolate-flavin CTC, but is also important for promoting the reduction of the N-terminal disulfide. Titration of E514A with NADPH led to a more pronounced thiolate-flavin CTC than was observed with the wild-type enzyme. This suggests that the transfer of electrons from the N-terminal redox-active dithiol to the C-terminal disulfide is impaired in this enzyme species, so that a greater fraction of the enzyme exists as EH$_2$(B). The loss of Glu-514, the epsilon-carboxyl in particular, and its proposed hydrogen bond to the delta nitrogen of His-509 (30), may force this imidazole ring to favor an increased electron density of the Cys-93 thiol on the acceptor flavin, thus leading to the observed energetically stronger thiolate-flavin CTC interaction. Mutations of both His-509 and Glu-514 lead to alterations of the equilibrium ratio of reaction intermediates and to impairment of the catalytic cycle, as corroborated by the analysis of the oxidative half-reactions (see below).

Stopped-flow studies showed that the wild-type and the two variant enzymes are rapidly reduced by NADPH to form the FADH$^-$-NADP$^+$ CTC (Table 3 and Figs. 2 and 3). The rates of the transfer of reducing equivalents from reduced flavin to the N-terminal disulfide with formation of the thiolate-flavin CTC (the second phase) and the subsequent interchange between the nascent N-terminal dithiol and the C-terminal disulfide are dramatically decreased when His-509 is substituted by glutamine. This was shown by the much slower third phase in Fig. 2B for H509Q. Although the third phase is thought to be primarily caused by formation of E$_{ox}$, this reaction cannot readily occur until EH$_2$(D) forms via the interchange reactions. The E514A variant shows a significant but less dramatic decrease in these phases compared with H509Q. The rates of the second and third phases of the reductive half-reaction for H509Q (Fig. 3, steps involving reduction of disulfides), were much more pH-dependent than those with wild-type, also consistent with the role of the dyad as the acid-base catalyst (Fig. 3).

In the reaction of EH$_4$ with PfTrx (the oxidative half-reaction), we attribute the fast phase to the reoxidation of EH$_4$ to EH$_2$ and the slow phases to the conversion of EH$_2$ to E$_{ox}$ (Fig. 4). The lack of dependence of the rate of the fast phase on the concentration of PfTrx can be explained by reference to Scheme 1. The binding of PfTrx to EH$_4$(B) could be very fast and tight so that only reactions following reoxidation of EH$_4$, i.e. Trx(SH)$_2$ dissociation and the establishment of equilibria among EH$_2$ species, which are first-order reactions, are observed. Thus, the rate of reoxidation would be independent of the Trx concentration (to avoid making Scheme 1 any more complex, the second half of the dithiol-disulfide interchange reaction between the reduced protein and PfTrx, and the dissociation of Trx were drawn as a single step). The fact that PfTrx only slowly oxidizes PfTrxR to EH$_2$ instead of E$_{ox}$ demonstrates that PfTrxR, like DmTrxR, cycles in catalysis between the EH$_4$ and EH$_2$ levels (25). During turnover very little E$_{ox}$ ever forms, and under most physiological conditions, the high NADPH concentration would rapidly reduce any E$_{ox}$ that formed back to EH$_2$.

The reaction of H509Q reduced with NADPH and reoxidized with PfTrx, was much slower than that of the wild-type PfTrxR, as shown in the inset of Fig. 5A; the half-time for the second phase, representing reoxidation of H509Q PfTrxR, was ~50 s, compared with ~0.3 s for wild-type enzyme. Nevertheless, H509Q was virtually fully reoxidized (Fig. 5A, inset). The first kinetic phase for H509Q, with a rate constant of 11 s$^{-1}$, represents only ~10% of the total extinction change at 490 nm, and, as mentioned under "Results," is attributed to dissociation of NADP$^+$ (Table 4). The major phase represents the reoxidation. Thus, these results show that the substitution of His-509 by glutamine severely retards both the oxidative and the reductive half-reactions of PfTrxR. Because both reactions involve dithiol-disulfide interchange reactions, these results are fully consistent with His-509 being the acid-base catalyst.

The oxidative half-reaction of E514A PfTrxR, reduced with NADPH and reoxidized with PfTrx, was ~20-fold slower than that of the wild-type protein, as shown in the inset of Fig. 5B and Table 4; the half-time for the phase representing reoxidation of E514A PfTrxR was ~6 s, compared with ~0.3 s for wild-type. Fig. 5B shows that in contrast to the H509Q enzyme, the E514A enzyme is only ~half-oxidized by excess PfTrx. It is possible that the loss of negative charge in this variant makes EH$_2$ a poorer reductant so that it cannot reduce PfTrx. The slow rate of reoxidation of EH$_4$ by PfTrx in the E514A variant also indicates the important role in the dithiol-disulfide interchange reaction between the N- and C-terminal cysteines (Scheme 1).

**Conclusions**—The dyad formed by His-509 and Glu-514 acts as the acid-base catalyst in PfTrxR and very likely the analogous pair has a similar function in all high-M$_r$ TrxRs. His-509 is the acid-base catalyst for transferring reducing equivalents from the N-terminal to the C-terminal cysteine pair. In addition, it stabilizes the thiolate-flavin CTC. Glu-514 appears to make His-509 a better catalyst and also alters the equilibrium of the dithiol-disulfide interchanges between EH$_2$(B) and EH$_2$(D). Because both mutations affect the dithiol-disulfide interchange...
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