Intersubunit Interactions in *Plasmodium falciparum* Thioredoxin Reductase*

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The thioredoxin redox system is composed of the NADPH-dependent homodimeric flavoprotein thioredoxin reductase (TrxR) and the 12-kDa protein thioredoxin. It is responsible for the reduction of disulfide bridges in proteins such as ribonucleotide reductase and several transcription factors. Furthermore, thioredoxin is involved in the detoxification of hydrogen peroxide and protects the cell against oxidative damage. There exist two classes of TrxRs: the high *M*ₘ and the low *M*ₙ proteins. The well characterized *Escherichia coli* TrxR represents a member of the low *M*ₙ class of proteins, whereas the mammalian, *Caenorhabditis elegans*, and *Plasmodium falciparum* proteins belong to the family of high *M*ₘ proteins. The primary structure of these proteins is very similar to that of glutathione reductase and lipoamide dehydrogenase. However, the high *M*ₘ TrxRs possess, in addition to their redox active N-terminal pair of cysteines, a pair of cysteine residues or a selenenylsulfide motif at their C terminus. These residues have been shown to be crucial for the reduction of thioredoxin. In this study we address the question whether the active site residues of *P. falciparum* TrxR are provided by one or both subunits. Differentially tagged wild-type and *P*/*TrxR mutant proteins were co-expressed in *E. coli* and the recombinant protein species were purified by affinity chromatography specific for the respective tags of the recombinant proteins. Co-expression of *P*/TrxR wild-type and mutant proteins resulted in the formation of three different protein species: homodimeric *P*/TrxR wild-type proteins, homodimeric mutant proteins, and heterodimers composed of one *P*/TrxR wild-type subunit and one *P*/TrxR mutant subunit. Co-expression of the double mutant *P*/TrxRC88A/C535A with *P*/TrxR wild-type generated an inactive heterodimer, which indicates that *P*/TrxR possesses intersubunit active sites. In addition, the data presented possibly imply a cooperative interaction between both active sites of *P*/TrxR.

Infection with *Plasmodium falciparum*, the causative agent of malaria tropica, is responsible for 2–3 million deaths per year. The malaria parasite spends part of its developmental life cycle in human erythrocytes where it is challenged with enhanced oxidative stress. Therefore the parasite needs efficient anti-oxidants to protect itself against damages, such as nucleic acid modifications, lipid peroxidation, or oxidation of thiol-containing proteins, caused by reactive oxygen species. The thioredoxin redox system, composed of the NADPH-dependent homodimeric thioredoxin reductase (TrxR)¹ and the 12-kDa protein thioredoxin (Trx) confers reduction of protein disulfides, ribonucleotide reductase being the most prominent example (1). Apart from this, thioredoxin interacts with a number of transcription factors in prokaryotic and eukaryotic cells, resulting in modified DNA binding activities and altered gene transcription (2). Another important function is the interaction of reduced thioredoxin with thioredoxin-dependent peroxidases, which detoxify reactive oxygen species and aid in the prevention of oxidative damage in the cell (3).

There exist two classes of TrxRs, the low *M*ₘ proteins are represented by the 35-kDa *Escherichia coli* TrxR; the high *M*ₙ proteins were identified in mammals, *P. falciparum*, and also the free living nematode *Caenorhabditis elegans* (4–7). The two classes of proteins do not only differ in their primary structure but also in their reaction mechanisms. *E. coli* TrxR achieves catalysis by a conformational change (8, 9), whereas high *M*ₙ TrxRs transfer reducing equivalents to their substrate by an intramolecular dithiol/disulfide interchange between the N-terminal redox active cysteines and the C-terminal pair of cysteines (10–12). The primary structure of *P*/TrxR resembles that of glutathione reductase and lipoamide dehydrogenase with respect to the location of the N-terminal redox active cysteine pair, and the occurrence of the C-terminal pair of cysteines is similar to mercuric ion reductase. In mercuric ion reductase it was shown that the C-terminal residues are involved in the catalytic process but are not redox active as in TrxRs (13, 14).

To achieve transfer of electrons from the N terminus to the C terminus, the redox active residues should be located in close proximity to each other. There are two possible conformations which allow this transfer: either by the formation of an intermolecular interaction between the two subunits of the dimeric protein or by an intrasubunit interaction where each of the subunits forms its own active site (Fig. 1). In mercuric ion reductase an intermolecular interaction between both subunits occurs (15). In glutathione reductase and lipoamide dehydrogenase the active sites are also formed by an interaction of both subunits; the redox

¹ The abbreviations used are: TrxR, thioredoxin reductase; Trx, thioredoxin; DTNB, 5,5'-dithiobis(2-nitrobenzoic acid); *P*/TrxR, *Plasmodium falciparum* thioredoxin reductase; TrxR, thioredoxin reductase; PCR, polymerase chain reaction; PAGE, polyacrylamide gel electrophoresis; WT, wild-type.
active cysteine residues are provided by one subunit, and the acid/base catalyst is provided by the second subunit (16).

To analyze the organization of the P/TrxR active sites, we decided to co-express wild-type and mutant P/TrxR (P/TrxRC88A, P/TrxRC535A, or P/TrxRC88AC535A) in E. coli and to separate the recombinant proteins. Using this method we expected to generate homodimeric enzymes consisting of wild-type or mutant subunits only and heterodimeric proteins consisting of one wild-type and one mutant subunit. Co-expressing single or double mutant P/TrxRs with P/TrxR wild-type and characterizing the recombinant protein species should give a clear picture of how the active sites of P/TrxR are formed and answer the question whether the homodimeric protein possesses intersubunit or intrasubunit active sites (Fig. 1).

EXPERIMENTAL PROCEDURES

Materials—E. coli thioredoxin was a kind gift from Professor Charles H. Williams, Jr. (Ann Arbor, MI). The expression vector pJC40 was a gift from Dr. Joachim Clos (Bernhard Nocht Institute, Hamburg, Germany). The expression vector pASK-IBA7 and StrepTactin-Sepharose were from the Institut für Bioanalytik (Göttingen, Germany). Hist- and Binds-Resin was from Novagen (Heidelberg, Germany). The plasmid pA/CYC184, restriction enzymes, T4 DNA ligase, and T4 DNA polymerase were obtained from New England Biolabs (Frankfurt am Main, Germany). Pfu polymerase and E. coli BLR (DE3) were from Stratagene (La Jolla, CA). The expression cassette of pJC40 was subcloned into pACYC184 as described under “Construction of Expression Plasmids.”

DNA Manipulation and Nucleotide Sequence Analyses—Routine DNA manipulations and transformations were performed as described by Sambrook et al. (17). The plasmids containing the genetically modified P/TrxR constructs were sequenced using the Sanger dideoxy-chain termination reaction for double-stranded DNA (17). The plasmids p/TrxR mutants P/TrxRC88A and P/TrxRC535A were generated using the mutagenic oligonucleotides sense 5'-GCTAAAAGGGAGGCTG-3' and antisense 5'-TGCACATTTTCTCACCCGCCCCCCTCCCCAT-3' and P/TrxRC88A in pJC40 as a template. The introduction of the second mutation was verified by nucleotide sequence analysis of the entire insert as described above.

FIG. 1. Possible organizations of the active sites of the dimeric P. falciparum thioredoxin reductase. A, intrasubunit active sites; two independent active sites are formed. The mutations of Cys88→Ala and Cys535→Ala are shown. B, intersubunit active site; residues of both subunits contribute to the formation of both active sites of the dimeric protein. The mutations of Cys88→Ala and Cys535→Ala are shown.

Construction of Expression Plasmids—To guarantee co-expression and co-existence of two individual plasmids in one E. coli cell, they need to have distinct origins of replication and selectable markers. We intended to use one plasmid containing an N-terminal Strep-tag and one plasmid containing an N-terminal His-tag, which would result in the formation of differentially tagged recombinant proteins. The His-tag plasmids (pSZ1, pSZ1TrxRC88A, pSZ1TrxRC535A, and pSZ1TrxRC88AC535A) were constructed as shown in Fig. 2. The plasmid pACYC184 (carrying the p15A origin of replication and a chloramphenicol acetyltransferase as selectable marker) was digested with Sall, and the 5'-overhang was filled in with T4 DNA polymerase according to standard methods (17). The expression cassette of pJC40 was isolated using DrdI, and the 3'-overhang was blunted with T4 DNA polymerase. The Sall-digested plasmid pACYC184 and the isolated JC40 expression cassette were digested with BstHI and ligated using T4 DNA ligase, resulting in the expression plasmid pSZ1. To obtain the expression plasmids pSZ1TrxRC88A, pSZ1TrxRC535A, and pSZ1TrxRC88AC535A, respectively, the P/TrxR mutants P/TrxRC88A, P/TrxRC535A, and P/TrxRC88AC535A were amplified by PCR using Pfu polymerase and plasmid DNA containing the respective mutant P/TrxR as template with the following PCR program: 95 °C (1 min) and 30 cycles of 95 °C (1 min), 50 °C (1 min), and 72 °C (3 min). The sense oligonucleotide 5'-GCACCCCGGGCCATGTGTAAGAAGATAAAAAGG-3' coding for the first 6 amino acids of P/TrxR contained a Smal restriction site. The antisense oligonucleotide 5'-GGGCCGGGCCCTTATCACCACCTTCCACCC-3'
encoding the last 6 amino acids of PfTrxR generated an ApaI restriction site to facilitate directional cloning. The expression plasmid pSZ1 and the gel-purified mutant PfTrxR inserts were double digested with SmaI and ApaI and ligated using T4 DNA ligase.

Wild-type PfTrxR was amplified by PCR using Pfu polymerase using the same PCR protocol as described above. The sense oligonucleotide 5'-GCCGCGGGTTCGGGCGGCTGAAAGATAAAAACG-3' coding for the first 6 amino acids and the antisense oligonucleotide 5'-GCCGCGGGGTTCGGGCGGCTGAAAGATAAAAACG-3' encoding the last 5 amino acids of PfTrxR both contained BsaI restriction sites. The purified PCR product (pTrxRWT) and the expression vector pASK-BBA7 (ColEl1 origin of replication, can be selected for with ampicillin) were ligated using T4 DNA ligase, resulting in pStrep/pTrxRWT.

**Expression and Purification of PfTrxR Homo- and Heterodimers—**

The expression vector pStrep/pTrxRWT was transformed into competent E. coli BLR (DE3). Subsequently the bacteria containing pStrep/pTrxRWT were made competent for co-transformation with pSZ1TrxRC88A, pSZ1TrxRC535A, or pSZ1TrxRC88AC535A (pSZ1Mut), respectively. An overnight culture of the freshly co-transformed bacteria was diluted 1:50 in 3 liters of fresh Luria Bertani-medium containing 50 μg ml⁻¹ ampicillin and 35 μg ml⁻¹ chloramphenicol and grown at 37 °C until the A₆₀₀ reached 0.5. Expression of wild-type protein (pStrep/pTrxRWT) was induced using 200 μg of anhydrotetracycline per liter of bacterial culture, and the temperature was reduced to 20 °C. Three hours later the expression of pSZ1Mut was induced by addition of 500 μM isopropyl β-D-thiogalactopyranoside, and the bacterial cultures were incubated overnight. The cells were harvested by centrifugation (4000 × g, 10 min). The bacterial pellet was resuspended in buffer W (100 mM Tris/HCl, pH 8.0, 1 mM EDTA), sonicated (Branson Sonifier 250), and the cell lysate was centrifuged at 100,000 × g for 1 h (Centrikon-T 1065, Kontron). The supernatant was applied to a 2 ml (lane 1) or 20 ml (lane 2) of Ni⁺⁺-chelating chromatography. This procedure resulted in the separation of three different protein species: 1) homodimeric Strep-tagged pTrxR, 2) homodimeric His-tagged pTrxR mutant proteins, and 3) heterodimeric Strep/His-tagged wild-type and mutant PfTrxR.

**RESULTS AND DISCUSSION**

**Expression and Purification of PfTrxR Homo- and Heterodimers—**

The specific activities of homo- (wild-type and mutant) and heterodimeric proteins were determined by the DTNB reduction assay using E. coli Trx and insulin (19). The DTNB reduction assay (1 ml, 20 °C) contained 100 mM potassium phosphate buffer, pH 7.5, 0.2 mM EDTA, 10 μM NADPH, and 3 mM DTNB. The change in absorbance was monitored at 412 nm (UVitron 932, Kontron). The E. coli Trx reduction assay (1 ml, 37 °C) consisted of 100 mM HEPES buffer, pH 7.5, 0.2 mM EDTA, 10 μM NADPH, 200 μM EDTA, and 100 μM E. coli Trx. The change in absorbance was measured at 340 nm.
**TABLE I**

| Proteins                        | E. coli Trx | DTNB               |
|---------------------------------|-------------|--------------------|
|                                 | Units/mg    | % WT activity | % expected | Units/mg     | % WT activity | % expected |
| Homo WT                         | 3.9 ± 0.5   | 100             | 100        | 4.2 ± 1.1   | 100           | 100        |
| Hetero                          | 2.2 ± 0.3   | 56              | 50         | 2.8 ± 0.1   | 67            | 50         |
| Homo C88A                       | 0.4 ± 0.1   | 10              | 0          | 0.1 ± 0.02  | 2             | 0          |
| Homo WT                         | 3.6 ± 0.9   | 100             | 100        | 5.6 ± 1.6   | 100           | 100        |
| Hetero C535A                    | 1.5 ± 0.5   | 42              | 50         | 3.1 ± 0.6   | 55            | 80         |
| Homo WT                         | 0.3 ± 0.1   | 8               | 0          | 3.6 ± 1.5   | 64            | 60         |
| Hetero                          | 0.4 ± 0.1   | 11              | 0          | 2.5 ± 0.6   | 58            | 30         |
| Homo C88AC535A                  | 0.2 ± 0.1   | 5               | 0          | 0.2 ± 0.08  | 5             | 0          |

**FIG. 5.** Intersubunit organization of *P. falciparum* thioredoxin reductase. A, Strep-tagged wild-type PfTrxR; B, His-tagged PfTrxRC88A/C535A; C, Strep/His-tagged heterodimer—residues of both subunits contribute to the formation of both active sites.

close proximity. There are two possible arrangements one can envisage for such an interaction. One of them would be an intramolecular interaction and thus the formation of two independent active sites in the homodimeric protein; the second would be an intermolecular interaction where amino acid residues are supplied by both subunits to form the active sites of the enzyme (Fig. 1). To investigate how the active sites of PfTrxR are formed, we co-expressed different PfTrxR species in *E. coli* BLR (DE3), separated the generated proteins from each other and determined their specific activities and $K_m$ values. Co-expression and subsequent purification of StrepPfTrxRWT with three constructs carrying different active site mutations resulted in the isolation of three different protein species: Strep-tagged wild-type homodimers, His-tagged mutant homodimers, and Strep/His-tagged wild-type/mutant heterodimers (Figs. 3 and 4). The protein concentrations of the three protein species in one preparation, however, was quite different. The homodimers were expressed at much higher levels than the heterodimeric proteins. There are several explanations for this phenomenon. It is possible that the heterodimers formed during co-expression are not very stable and are degraded more rapidly by the bacterial cell than the homodimeric proteins. Another reason might be the synthesis of monomers that do not dimerize co-translationally and are therefore degraded rapidly by the bacterial cell. It has been shown previously that monomeric mouse glutathione reductase is degraded rapidly by bacterial cells (22). The generation of *E. coli* glutathione reductase hybrid protein species resulted in high yields of proteins, and there was no mention about a differential expression pattern concerning the different protein species that were generated (23). Similarly, the co-expression of mercuric ion reductase hybrids apparently did not result in different yields of the hybrid proteins (15).

**Specific Activities of Recombinant PfTrxR Homo- and Heterodimers**—The specific activities of the co-expressions with wild-type and three mutant PfTrxRs were determined using two different assay systems. The results are summarized in Table I. The specific activities of the Strep-tagged wild-type PfTrxR are comparable with those of preparations of nontagged recombinant PfTrxR in both assays (4.5 ± 1.5 units mg$^{-1}$ without tag versus 3.9 ± 0.5 units mg$^{-1}$ with a Strep-tag using 100 μM *E. coli* thioredoxin and 6.2 ± 0.8 units mg$^{-1}$ versus 5.6 ± 1.6 units mg$^{-1}$ using DTNB).

The activities of PfTrxRWT and PfTrxRC88A with *E. coli* thioredoxin are in good accordance with the expected results regardless whether the active sites of PfTrxR are formed in an intersubunit or intrasubunit interaction. The homodimers are either 100% active (PfTrxRWT, 3.9 units mg$^{-1}$) or show a very low activity of 0.4 unit mg$^{-1}$ (PfTrxRC88A). It has been shown previously that the mutation of Cys$^{535}$ into alanine resulted in a loss of DTNB and thioredoxin reducing activity and thus that this residue is essential for the catalytic activity of PfTrxR (18). The hybrid protein formed between PfTrxRWT and PfTrxRC88A has 56% of wild-type activity, which suggests that the protein possesses one fully active catalytic site and one inactive catalytic site. Using DTNB as a substrate the results are similar. The heterodimer formed between wild-type and mutant subunit exhibits similar kinetic parameters to those of the wild-type protein ($K_m$ PfTrxRWT/mutant heterodimer: 384 ± 59 μM versus $K_m$ PfTrxRWT: 429 ± 57 μM), indicating that the C88A mutation is only acting on one of the active sites, and no interaction is transmitted across the interface to the second active site.

The alteration of Cys$^{535}$ into alanine rendered the protein
inactivating toward *E. coli* thioredoxin, and the DTNB reduction by this mutant was decreased to about 60% of wild-type activity (19). In accordance, *P*/*TrxRWT* in this co-expression experiment is fully active, and the C535A mutant homodimer is almost completely inactive using *E. coli* thioredoxin as a substrate and maintains about 60% of its DTNB reducing activity. The heterodimer between *P*/*TrxRWT* and *P*/*TrxRC535A* subunits maintains only 42% of residual activity rather than 50%, for the reduction of *E. coli* thioredoxin. This slight discrepancy could possibly be explained by contamination of this protein fraction with inactive homodimers of mutant protein, which may occur during the purification procedure. The results obtained in the DTNB reduction assay are, however, more difficult to explain. The heterodimers between *P*/*TrxRWT* and *P*/*TrxRC535A* show a residual activity of 55%, which is much lower than the expected 80%, taking into account that one fully active and one slightly impaired catalytic site (60% residual activity) are formed in this enzyme hybrid (Table I). Interestingly the *Kₐ* value for DTNB reduction is higher for the wild-type protein (678 ± 1 μM versus 429 ± 57 μM) and lower than for the *P*/*TrxRWT* homodimer (678 ± 1 μM versus 800 ± 22 μM). These results suggest that the alteration of Cys⁵³⁵ in one active site possibly results in subtle changes in the reactivity of the second active site, which may interfere with DTNB and also thioredoxin reduction. An asymmetry in the catalytic activity of the two active sites of *P*/*TrxR* occurring during the interchange of electrons between the N-terminal cysteine residues and the C-terminal cysteine residues has been previously proposed by Wang *et al.* (24), and asymmetry of the active sites occurs in several other members of this enzyme family (25–27). The data presented here support this hypothesis, and these possible cooperative interactions will be investigated in more detail in future studies.

The key experiment that allows us to draw final conclusions about the formation of the *P*/*TrxR* active sites was the co-expression of *P*/*TrxRWT* with the double mutant *P*/*TrxRC88AC535A*. Both enzymatic assays show the results expected when the active sites of *P*/*TrxR* are formed by an intersubunit interaction between both subunits of the dimeric protein (Fig. 5). The heterodimers between *P*/*TrxRWT* and *P*/*TrxRC88AC535A* were inactive and the homodimeric mutant proteins also did not show an appreciable enzymatic activity with thioredoxin (Table I). Therefore it can be concluded that the interaction of the N-terminal cysteine residues Cys⁸₈ and Cys⁹₉ with the protein-bound FAD occurs on one subunit, and the reducing equivalents are then transferred to the C-terminal cysteine residues Cys⁵³⁵ and Cys⁵⁴⁰ of the second subunit. The thiolate anions are most likely stabilized by the acid/base His⁶⁰⁹ also provided by the second subunit (18). This conformation resembles those of the glutathione reductase, lipoamide dehydrogenase, and mercuric ion reductase. Perham and co-workers (23, 28) have demonstrated a similar interaction between the subunits in *E. coli* glutathione reductase by separating homo- and heterodimers by engineering an arginine-tag to one of the subunits and co-expressing the arginine-tagged and unmodified proteins in *E. coli*. Neither of the hybrid proteins formed in this study had any influence on the kinetic parameters, indicating that in glutathione reductase no cooperative effects between the two subunits occur (29). In mercuric ion reductase, which like *P*/*TrxR* possesses an additional pair of Cys residues at the C-terminus, intersubunit active sites are formed (15). A cooperative interaction between both active sites has been suggested in mercuric ion reductase (25) similar to our observations on *P*/*TrxR* described in Wang *et al.* (24) and in this study. The generation of heterodimeric *P*/*TrxR* protein species will facilitate future studies on the catalytic mechanism of the protein, such as potential cooperative interactions between the active sites during catalysis (25, 26, 30). In addition the knowledge of the architecture of the *P*/*TrxR* catalytic centers allows the overexpression of dominant negative mutants in *P. falciparum*, which will help to determine the biological role of the protein in the parasites. Ultimately this approach will aid to validate and assess *P. falciparum* thioredoxin reductase as a target for a chemotherapeutic intervention of malaria.

REFERENCES

1. Holmgren, A. (1985) *Annu. Rev. Biochem.* 54, 237–271.
2. Kamata, H. & Hirata, H. (1999) *Cell Signal.* 11, 1–14.
3. Netto, L. E. S., Chae, H. Z., Kang, S. W., Rhee, S. G. & Stadtman, E. R. (1996) *J. Biol. Chem.* 271, 15315–15321.
4. Russel, M. & Model, P. (1988) *J. Biol. Chem.* 263, 9015–9019.
5. Gasdaska, P. Y., Gasdaska, J. R., Cochran, S. & Powis, G. (1995) *FEBS Lett.* 373, 5–9.
6. Muller, S., Gilberger, T. W., Farber, P. M., Becker, K., Schirmer, R. H. & Walter, B. (1996) *Mol. Biochem. Parasitol.* 80, 215–219.
7. Buettner, C., Harney, J. W. & Berry, M. J. (1999) *J. Biol. Chem.* 274, 21598–21602.
8. Williams, C. H., Jr. (1995) *FASEB J.* 9, 1267–1276.
9. Lennon, B. W., Williams, C. H., Jr. & Ludwig, M. L. (2000) *Science* 289, 1190–1194.
10. Arscott, L. D., Gromer, S., Schirmer, R. H., Becker, K. & Williams, C. H., Jr. (1997) *Proc. Natl. Acad. Sci. U. S. A.* 94, 3621–3626.
11. Wang, P. F., Arscott, L. D., Gilberger, T. W., Muller, S. & Williams, C. H., Jr. (1999) *Biochemistry* 38, 3187–3196.
12. Zhong, L., Arner, E. S. & Holmgren, A. (2000) *Proc. Natl. Acad. Sci. U. S. A.* 97, 5854–5859.
13. Moore, M. J. & Walsh, C. T. (1989) *Biochemistry* 28, 1183–1194.
14. Moore, M. J., Miller, S. M. & Walsh, C. T. (1992) *Biochemistry* 31, 1677–1685.
15. Distefano, M. D., Moore, M. J. & Walsh, C. T. (1990) *Biochemistry* 29, 2703–2713.
16. Thieme, R., Pai, E. F., Schirmer, R. H. & Schulz, G. E. (1981) *J. Mol. Biol.* 152, 763–782.
17. Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, 2nd ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
18. Gilberger, T. W., Walter, R. D. & Muller, S. (1997) *J. Biol. Chem.* 272, 29684–29689.
19. Gilberger, T. W., Bergmann, B., Walter, R. D. & Muller, S. (1998) *FEBS Lett.* 425, 407–410.
20. Bradford, M. M. (1976) *Anal. Biochem.* 72, 248–254.
21. Gladyshev, V. N., Jeang, K. T. & Stadtman, T. C. (1996) *Proc. Natl. Acad. Sci. U. S. A.* 93, 6146–6151.
22. Nordhoff, A., Bucheler, U. S., Werner, D. & Schirmer, R. H. (1993) *Biochemistry* 32, 4060–4066.
23. Deonarain, M. P., Scritton, N. S. & Perham, R. N. (1992) *Biochemistry* 31, 1498–1504.
24. Wang, P. F., Arscott, D. L., Gilberger, T. W., Muller, S. & Williams, C. H., Jr. (1999) in *Flavins and Flavoproteins* (Ghida, S., Korneck, P. M. H., Macheraux, P., and Sund, H., eds) pp. 695–698, Agency for Scientific Publications, Berlin.
25. Muller, S. M., Massey, V., Williams, C. H., Jr., Ballou, D. P. & Walsh, C. T. (1991) *Biochemistry* 30, 2600–2612.
26. Luba, J., Charrier, V. & Clairborne, A. (1999) *Biochemistry* 38, 2725–2737.
27. Mallett, T. C., Parsonage, D. & Clairborne, A. (1999) *Biochemistry* 38, 3000–3011.
28. Deonarain, M. P., Scritton, N. S. & Perham, R. N. (1992) *Biochemistry* 31, 1491–1497.
29. Scritton, N. S., Berry, A., Deonarain, M. P. & Perham, R. N. (1990) *Proc. R. Soc. Lond. B Biol. Sci.* 242, 217–224.
30. Mallett, T. C. & Clairborne, A. (1998) *Biochemistry* 37, 8790–8802.