Recombinant *Plasmodium falciparum* glutathione reductase is inhibited by the antimalarial dye methylene blue

P.M. Färber[a,*], L.D. Arscott[b], C.H. Williams Jr.[b], K. Becker[a], R.H. Schirmer[a]

[a] *Biochemie-Zentrum der Universität Heidelberg, 5 OG, Im Neuenheimer Feld 328, D-69120 Heidelberg, Germany*
[b] *Department of Veterans Affairs Medical Center and Department of Biological Chemistry, University of Michigan, Ann Arbor, MI 48105, USA*

Received 21 November 1997; revised version received 7 January 1998

Abstract ***Plasmodium falciparum*** **glutathione reductase (PfGR) has emerged as a drug target against tropical malaria. Here we report the expression of PfGR in** ***Escherichia coli*** **SG5(DE3) and isolation procedures for this protein. Recombinant PfGR does not differ from the authentic enzyme in its enzymic properties, the turnover number being 9900 min^{-1}. The dimeric flavoenzyme exhibits redox-dependent absorption spectra; the single tryptophan residue (per 57.2 kDa subunit) is strongly fluorescent. PfGR can be inhibited by the antimalarial drug methylene blue at therapeutic concentrations; the K_i for non-competitive inhibition is 6.4 μM. The sensitivity to methylene blue is observed also at high ionic strength so that, by analogy to human GR, analysis of crystalline enzyme-drug complexes can be envisaged.**

Key words: Malaria; Drug target; Disulfide reductase; Flavoenzyme; Phenothiazine; Tryptophan fluorescence

1. Introduction

The clinical manifestations of tropical malaria are caused by the multiplication and differentiation of the protozoan parasite *Plasmodium falciparum* in human erythrocytes. Malarial parasites appear to be more sensitive to reactive oxygen species than their host cells [1]. Inherited or drug-induced deficiency of antioxidative erythrocyte enzymes such as glucose-6-phosphate dehydrogenase [2,3] or glutathione reductase [4,5] appear to protect from severe forms of falciparum malaria. Glutathione reductase (GR), a dimeric flavoenzyme, catalyses the reduction of glutathione disulfide using NADPH as a source of reducing equivalents: $GSSG+NADPH+H^+ = 2GSH+NADP^+$. In the cytosol the enzyme maintains a [GSH]/[GSSG] ratio of ≥100. Glutathione in its reduced form represents the most abundant intracellular non-protein thiol. It is involved in a broad range of functions such as protection of biomolecules from oxidative damage, detoxification of xenobiotics, and regulation of enzyme activity [6–9].

Both *P. falciparum* GR and human erythrocyte GR play crucial roles for the intraerythrocytic growth of the parasite. Human GR is a well characterized protein; the catalytic cycle as well as the binding mode of drugs and other ligands have been studied in great detail [4,6,7,10–12]. *P. falciparum* GR (PfGR) has recently come into focus as a drug target. The enzyme was purified from parasitized red blood cells but only in μg quantities [13]. To promote structural and functional studies on this protein, the gene of PfGR was isolated [14]. As reported here, recombinant PfGR (rPfGR) was expressed, purified and characterized, in particular as a target of methylene blue. This antimalarial compound, the first chemotherapeutic agent to be successfully used in humans [15,16], is known to interfere with the glutathione metabolism of parasitized erythrocytes [17].

*Corresponding author. Fax: +49 (6221) 54 5586.
E-mail: Schirmer@novsrv1.pio1.uni-heidelberg.de

Abbreviations: E_{ox}, oxidized form of glutathione reductase containing an active site disulfide; EH_2, 2-electron reduced glutathione reductase containing an active site dithiol; GR, glutathione reductase (EC 1.6.4.2); GSH, glutathione (reduced state); GSSG, glutathione disulfide; IPTG, isopropyl-β-D-thiogalactopyranoside; ORF, open reading frame; *P. falciparum*, *Plasmodium falciparum*; RACE, rapid amplification of cDNA ends; rPfGR, recombinant *Plasmodium falciparum* glutathione reductase

2. Materials and methods

2.1. Materials and assay systems

E. coli SG5(DE3) was a kind gift of Dr. Sylke Müller, Bernhard-Nocht-Institute, Hamburg. Authentic PfGR (5 μg from 1 g erythrocytes containing *P. falciparum* FBRC schizonts) was isolated as previously reported [13]. Recombinant human GR was purified and assayed as described [12]. Expression vector pET22b+ was from Novagen, *Tth* DNA polymerase from Boehringer Mannheim, and restriction enzymes were from New England Biolabs. Superose 12 and 2′,5′-ADP-Sepharose were purchased from Pharmacia and NADPH, GSSG, and IPTG from Biomol. Methylene blue (Fluka) was used as a 10 mM stock solution in H_2O. Vivaspin concentrator units were from Greiner.

Protein in crude fractions was estimated by absorbance measurements at 228.5 nm and 234.5 nm according to Ehresman et al. [18]. For purified PfGR in the E_{ox} form, a solution of 1 mg/ml was found to have an absorbance of 0.205 at 461 nm and of 1.43 at 275 nm. These calibrations were performed by correlation with the absorbance coefficient of E_{ox} which is 11.7 mM^{-1} cm^{-1} at 461 nm per 57.2 kDa subunit (see Section 2.5).

2.2. Construction of the expression vector pG11

The fragments of the PfGR gene present in the plasmid constructs pG1, pG2, pG3, and pG4 described in [14] were fused by consecutive ligation into vector pT7 Blue (Novagen), taking advantage of unique restriction sites for *Hin*cII, *Nhe*I, and *Spe*I in the ORF, to finally obtain construct pTG1-4. The 5′-end was obtained by 5′-RACE-PCR on a *Tth* DNA polymerase-synthesized cDNA (T 6.1, see [14]); the oligonucleotide primer G2 binds downstream the unique *Hin*cII site (ATCGATCCAACGTTGACACACGTTC), whereas primer SN5 (ACGTAGCATGCCATATGGTTTACGATTTAATTG-TAATTG) introduces *Sph*I and *Nde*I restriction sites juxtaposed to the start codon. The PCR product was digested with *Sph*I and *Hin*cII and ligated into pT1-4, resulting in the complete ORF of PfGR in the plasmid pTG1-5. To remove the 3′-non-translated region of the cDNA, pTG1-5 was digested with *Sty*I and *Sac*I and recircularized by ligation of the hybridized oligonucleotides TGATGGATGAAAT-GACTCGAGGAGCT and CACCCTCGAGTCATTTCATC, thereby introducing an *Xho*I site upstream the *Sac*I site. From this construct, pTG, the complete ORF was excised by a *Nde*I-*Xho*I digest and inserted into plasmid pET22b+, resulting in pG11 which encodes the protein of 500 residues (N-terminal Met+499 residues [14]) under the control of a T7 RNA polymerase promoter. The plasmid was then introduced into *E. coli* SG5(DE3), a GR-deficient strain transformed with phage DE3 to allow T7 DNA polymerase-dependent expression.

PII S0014-5793(98)00031-3

2.3. Purification of recombinant PfGR

Plasmid-carrying SG5(DE3) cells were grown in Luria-Bertani medium containing 50 μg/ml carbenicillin to an apparent $OD_{600\ nm} > 0.5$. PfGR expression was then induced by 1 mM IPTG. After 12 h at 25°C the cells were harvested by centrifugation. The resulting bacterial pellet (appr. 7 g) was resuspended in lysing solution (50 mM Tris-HCl, 150 mM NaCl, 10% glycerol, 0.03% Triton X-100, 1 mg/ml lysozyme, 10 μM phenylmethylsulfonyl fluoride, 10 μg/ml DNase I, pH 7.5) and subjected to sonication.

After clearing centrifugation, the supernatant was saturated to 80% with ammonium sulfate and left for 12 h at 4°C. The resulting precipitate was collected by centrifugation, and the pellet was dialysed exhaustively at 4°C against 25 mM potassium phosphate, 1 mM EDTA, pH 7.0. The retentate was then applied to a 5 ml 2′,5′-ADP-Sepharose column equilibrated with the same buffer. This column was washed and eluted as described for authentic PfGR [13]. Fractions with PfGR activity were pooled, and 1 mM GSSG was added in order to oxidize the NADPH used for eluting the enzyme. The pool was then concentrated and washed with 25 mM potassium phosphate, 1 mM EDTA, pH 6.9, or 50 mM sodium phosphate, pH 6.9, respectively, using Vivaspin concentrator units. As judged by silver-stained SDS-PAGE the resulting protein was >98% pure (data not shown).

In order to fully saturate PfGR with its prosthetic group FAD, 1 ml protein solution (E_{ox} with an absorbance of 0.360 at $\lambda_{max,vis} = 461$ nm in 50 mM sodium phosphate, pH 6.9) was titrated with 100 μM FAD in the same buffer. 0.5 μl aliquots were removed after each FAD addition for measuring the enzyme activity. When more than 0.75 nmol FAD had been added the enzyme activity did not increase further and the spectrum of the solution indicated the presence of free FAD. It was concluded that the batch of purified enzyme contained 2.4% apoprotein, and FAD was supplemented to give a PfGR solution which contained 100 ± 0.3% holoenzyme, the absorbance at 461 nm being 0.369.

2.4. Steady state kinetics and inhibition studies

Glutathione reductase activity was assayed at 25°C with 100 μM NADPH and 1 mM GSSG in assay buffer (47 mM potassium phosphate, 200 mM KCl, 1 mM EDTA, pH 6.9) according to published procedures [19]. The enzyme-catalyzed conversion of NADPH to $NADP^+$ was followed spectrophotometrically (ε at 340 nm = 6.22 $mM^{-1}\ cm^{-1}$). One enzyme unit is defined as the GSSG-dependent oxidation of 1 μmol NADPH per min.

The inhibitory effect of methylene blue on recombinant PfGR, authentic PfGR [13] and human GR [12] was studied as follows. Methylene blue and NADPH were added first to the assay buffer and the decrease of absorbance ($\Delta A1/\Delta t$) was monitored for 1 min. This accounted for the spontaneous reaction of NADPH with methylene blue which was significant at drug concentrations above 10 μM. Subsequently GR (appr. 10 mU) was added and the ensuing $\Delta A2/\Delta t$ was followed for another minute. $\Delta A2/\Delta t$ was found not to differ appreciably from $\Delta A1/\Delta t$ indicating that GR has negligible methylene blue reduction activity. The glutathione disulfide reduction reaction was started with GSSG and yielded $\Delta A3/\Delta t$. After correction for dilution, the glutathione reductase activity was proportional to (ΔA3/min − ΔA2/min). The possibility of irreversible inhibition was tested by incubating PfGR at 2 U/ml (170 nM) for 30 min at 4°C and 25°C with 10, 100 or 500 μM methylene blue, and then assaying aliquots for enzyme activity. These incubations were carried out at pH 6.9 and pH 7.4 in the presence and absence of 100 μM NADPH.

2.5. Absorbance and fluorescence measurements

The ε-value at $\lambda_{max,vis}$ of recombinant PfGR in the E_{ox} form was computed on the basis of the amount of FAD separated from the protein by incubation with 0.2% SDS in 50 mM sodium phosphate buffer, pH 6.9, for 60 min at 45°C in the dark [20]. From 1 ml holoenzyme with an absorbance of 0.369 at 461 nm (see Section 2.3) 31.5 nmol FAD was released. This corresponds to an ε of 11.7 $mM^{-1}\ cm^{-1}$ for the holoenzyme. When determining FAD we took into account that, in the presence of 0.2% SDS, free FAD has a millimolar absorbance coefficient of 11.4 $mM^{-1}\ cm^{-1}$ at 448 nm and that 3% of the released FAD is thermally destroyed during the incubation at 45°C.

Absorption spectroscopy of PfGR under anaerobic conditions was carried out using the methods detailed for *E. coli* glutathione reductase [21]. After recording the spectrum of PfGR in oxidized form (E_{ox}; 9.5 nmol enzyme subunit in 1 ml 50 mM potassium phosphate, 2 mM EDTA, pH 7.45 at 25°C), the enzyme was converted to the EH_2 form using sodium borohydride (150 nmol dissolved in 2 ml 0.2 M NaOH) as a reductant. $NaBH_4$ hydrolysis ($t_{1/2} = 20$ s at pH 7.45) did not interfere with the reduction of PfGR which was complete in less than 1 min. The spectra at pH 6.9 exhibited the same characteristics as at pH 7.45. However, a 60-fold excess of sodium borohydride had to be used because of the 7-fold higher hydrolysis rate at pH 6.9 [22].

Fluorescence measurements were conducted according to Mulrooney [23]. For the emission spectra of FAD, both free and protein-bound, the excitation was set at 380 nm while the observed peak of relative fluorescence for both was at 540 nm. Tryptophan, both free and protein-bound, was excited at 295 nm; the emission peaks were at 330 nm and 340 nm, respectively. In addition to this blue shift [24], the fluorescence of the enzyme's unique Trp residue was enhanced 2-fold when compared with the free amino acid.

3. Results and discussion

3.1. Expression and purification of rPfGR

Recently we have characterized the PfGR gene of the *P. falciparum* strain K1 (Thailand) [14]. As the gene carries two introns, RT-PCR on parasitic mRNA from erythrocytic stages was used to obtain the 5′-end of the open reading frame. Expecting a solid stem structure of the mRNA, *Tth* DNA polymerase was used as this enzyme exhibits reverse transcriptase activity at 65°C. By several cloning steps, the resulting PCR product and the inserts of pG1–pG4 were fused to gain the complete ORF of PfGR in plasmid pTG. Its insert was excised and ligated into vector pET22b+, and the resulting construct pG11 was transformed into the glutathione reductase-deficient *E. coli* strain SG5(DE3). The expressed protein, recombinant PfGR, was purified to homogeneity and demonstrated high enzymatic activity (Table 1).

3.2. Characterization of recombinant PfGR

Systematic studies on a large malarial protein as a drug target are hampered by the following difficulties. The authentic protein can be isolated only in small amounts from the parasite and tends to be contaminated with host cell proteins of similar function. Furthermore, recombinant parasite genes often resist functional expression in heterologous systems. As reported here, it was possible to obtain *P. falciparum* glutathione reductase in good yield, and its enzymic properties were found to be practically indistinguishable from those of

Table 1
Isolation procedure for recombinant PfGR from a 1 l culture containing 7 g *E. coli* SG5(DE3) transformed with pG11

Fraction	Volume (ml)	Total PfGR (units)	Total protein (mg)	Spec. activity (units/mg)	Purification (-fold)	Yield (%)
Bacterial extract	20.0	1650	1120	1.47	1	100
Pellet after 80% $(NH_4)_2SO_4$; dialysed	24.0	1490	402	3.70	2.52	90.3
Eluate from 2′,5′-ADP-Sepharose (after concentration)	1.0	1160	6.70	173	118	70.3

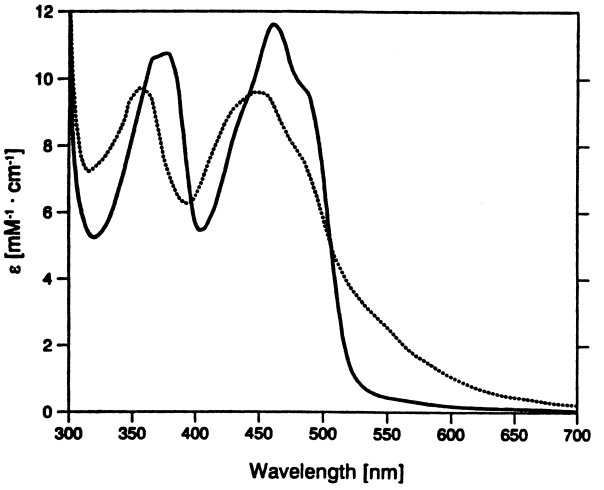

Fig. 1. Absorption spectra of PfGR. The measurements were conducted under anaerobic conditions with 9.6 μM enzyme in 50 mM potassium phosphate, 2 mM EDTA, pH 7.45 at 25°C [21]. The solid line represents E_{ox}, the yellow oxidized enzyme species, and the dotted line EH_2, the 2-electron reduced orange-colored enzyme species which was obtained from E_{ox} using sodium borohydride. The spectra of E_{ox} and EH_2 at pH 6.9 in 50 mM phosphate-2 mM EDTA buffer show the same characteristics as at pH 7.45 (data not shown). EH_2, possibly complexed with NADPH and carrying glutathione as a mixed disulfide [33] is the predominant form of glutathione reductase in vivo [7].

the authentic enzyme. Physicochemical studies on rPfGR were conducted in comparison with the human host enzyme (Table 2). The two enzymes had, for instance, comparable characteristics when subjected to gel filtration on Superose 12; this indicates that rPfGR is a homodimeric enzyme. As detailed in Section 2, the quantitation of rPfGR was based on the molar absorbance of the flavoenzyme in the E_{ox} form at 461 nm (Table 2). The absorption spectra of rPfGR in the oxidized form E_{ox} and the 2-electron reduced form EH_2 (Fig. 1) exhibit the typical characteristics of the pyridine nucleotide-disulfide oxidoreductase family [6]. Table 2 also includes data on the fluorescence of the flavin and the unique tryptophan residue of PfGR. These fluorescence properties can be used for monitoring changes in the protein induced by ligand binding and by mutations [24].

3.3. Inhibition of PfGR by methylene blue

When 10 μM methylene blue was present in the assay mixture the activity of rPfGR was 60% lower than in the control assay. This inhibition did not show any time dependence and was completely reversible by dilution. Also, preincubation of rPfGR at 2 U/ml (= 0.17 μM) with up to 500 μM methylene blue – in the absence or presence of 100 μM NADPH, at pH 6.9 or pH 7.4 – did not lead to irreversible inactivation. Other inhibitors such as carmustine are known to modify the NADPH reduced enzyme irreversibly [25].

The K_m-values for the substrates NADPH and GSSG were found to be unchanged in the presence of methylene blue. Assuming non-competitive inhibition, the K_i-values of methylene blue were 6.4 (± 0.3) μM for the parasite enzyme and 16.1 (± 0.8) μM for human GR (Fig. 2).

Methylene blue is an approved drug. Common indications are septic shock [26] – methylene blue appears to inhibit enzymes of NO metabolism [27] – and certain forms of methemoglobinemia [28]. Against malaria it has been used only on sporadic occasions [15,16]. At the therapeutic blood concentration of approximately 50 μM, a number of other molecular targets in *P. falciparum* such as the heme-polymerization reaction [29] might be affected. Glutathione reductase is the first molecule to be identified as a target of methylene blue in the parasite. Taking into account the specific accumulation of methylene blue in the parasites [16,30], the inhibition of PfGR (Fig. 2) is likely to contribute significantly to the cytotoxic effects. Consequently the phenothiazine structure of methylene blue [16] will be included in the lead compounds for the rational development of therapeutic GR inhibitors [4,11,31]. Work on the crystallization of the GR-methylene blue complexes is in progress. Under the conditions used for analyzing human GR crystals by X-ray diffraction (0.9 M ammonium sulfate in assay buffer), the apparent K_i-value of PfGR for methylene blue was found to be 23 μM while the K_m-values for NADPH and GSSG increased to about 100 μM. This suggests that the structures of catalytically competent PfGR as well as methylene blue-inhibited PfGR can be

Table 2
Comparison of recombinant *Plasmodium falciparum* GR with human host GR

Protein	PfGR	Human GR
M_r	114.3 kDa	104.8 kDa
Subunit M_r	57 155 Da [6]	52 395 Da
Prosthetic group	FAD	FAD
$\lambda_{max,vis}$ for E_{ox}	461 nm	463 nm
ε at $\lambda_{max,vis}$ for E_{ox}	11.7 mM^{-1} cm^{-1}	11.3 mM^{-1} cm^{-1}
A at 280 nm/A at $\lambda_{max,vis}$ for E_{ox}	6.35	6.7
Fluorescence of GR-bound vs. free compound FAD	≥12-fold quenching	≥20-fold quenching
W 497, the single Trp of PfGR	2-fold enhancement	does not apply
K_m for GSSG	83 μM	65 μM
K_m for NADPH	8.3 μM	8.5 μM
Activity with NADH/activity with NADPH	< 0.04	0.05
Turnover number	9900 min^{-1}	12 600 min^{-1}
Specific activity	173 U/mg	240 U/mg
Optimal pH for enzyme activity	6.9–7.2	6.7–6.9
K_i for methylene blue	6.4 μM	16 μM
- in the presence of 0.9 M $(NH_4)_2SO_4$	23 μM	34 μM

Apart from the K_i for methylene blue, the data for human GR were taken from [7,10–12]. Recombinant PfGR was found not to differ in its enzymic properties from the authentic enzyme. The somewhat lower K_m-values, namely 4.8 μM for NADPH and 69 μM for GSSG reported previously for authentic PfGR [13], could not be reproduced in the present study where more enzyme purified from *P. falciparum* was available for kinetic studies.

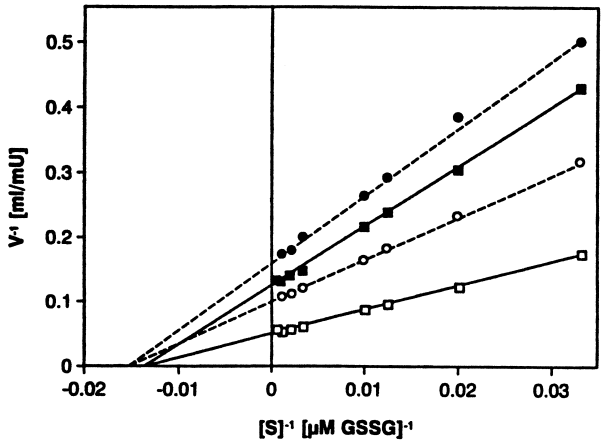

Fig. 2. Inhibition of PfGR and human GR by 10 μM methylene blue. As shown in the Lineweaver-Burk diagram for GSSG as a substrate, methylene blue is a non-competitive inhibitor of GR (open squares, PfGR; open circles, hGR; filled squares and circles, PfGR and hGR, respectively, in the presence of 10 μM methylene blue).

studied by crystallographic methods [10,11]. It will be interesting to see if the drug binds in the intersubunit cavity [10] as it is the case for most known tricyclic inhibitors of human GR [11,31,32]. When modelling the amino acid sequence of PfGR [14] into the structure of human GR [10], only 32% identity was found for the residues lining this cavity. In conclusion, structural comparisons of PfGR with human GR, particularly those distinctions responsible for the different inhibition by methylene blue, are expected to serve as an approach to the development of selective drugs.

Acknowledgements: The excellent technical assistance of Irene König is gratefully acknowledged. Dr. Scott Mulrooney communicated fluorescence results prior to publication. We thank Petra Krapp for her help with the manuscript. This research was supported by the Department of Veterans Affairs, by the National Institute of General Medical Sciences Grant GM21444, by the Fonds National Suisse (NF 31-29969), by the Fonds der Chemischen Industrie, and by the Bundesministerium für Forschung und Technologie (Research Focus Tropical Medicine, 01 KA 9301).

References

[1] Hunt, N.H. and Stocker, R. (1990) Blood Cells 16, 499–526.
[2] Golenser, J. and Chevion, M. (1989) Semin. Hematol. 26, 313–325.
[3] Scott, M.D., Wagner, T.C. and Chiu, D.T.Y. (1993) Biochim. Biophys. Acta 1181, 163–168.
[4] Schirmer, R.H., Müller, J.G. and Krauth-Siegel, R.L. (1995) Angew. Chem. Int. Ed. Engl. 34, 141–154.
[5] Anderson, B.B., Corda, L., Perry, G.M., Pilato, D., Ginberte, M. and Vullo, C. (1995) Am. J. Hum. Genet. 57, 674–681.
[6] Williams Jr., C.H. (1992) in: Chemistry and Biochemistry of Flavoenzymes (Müller, F., Ed.) vol. III, pp. 121–211, CRC, Boca Raton, FL.
[7] Schirmer, R.H., Krauth-Siegel, R.L. and Schulz, G.E. (1989) in: Coenzymes and Cofactors (Dolphin, D., Poulson, R. and Avramovic, O., Eds.) vol. IIIA, pp. 553–596, John Wiley and Sons, New York.
[8] Penninckx, M.J. and Elskens, M.T. (1993) Adv. Microbiol. Physiol. 34, 240–255.
[9] Cooper, A.J.L. and Kristal, B.S. (1997) Biol. Chem. 378, 793–802.
[10] Karplus, P.A. and Schulz, G.E. (1987) J. Mol. Biol. 195, 701–729.
[11] Savvides, S.N. and Karplus, P.A. (1996) J. Biol. Chem. 271, 8101–8107.
[12] Nordhoff, A., Bücheler, U.S., Werner, D. and Schirmer, R.H. (1993) Biochemistry 32, 4060–4066.
[13] Krauth-Siegel, R.L., Müller, J.G., Lottspeich, F. and Schirmer, R.H. (1995) Eur. J. Biochem. 235, 345–350.
[14] Färber, P.M., Becker, K., Müller, S., Schirmer, R.H. and Franklin, R.M. (1996) Eur. J. Biochem. 239, 655–661.
[15] Guttmann, P. and Ehrlich, P. (1891) Berl. Klin. Wochenschr. 28, 953–956.
[16] Vennerstrom, J.L., Makler, M.T., Angerhofer, C.K. and Williams, J.A. (1995) Antimicrob. Agents Chemother. 39, 2671–2677.
[17] Kelner, M.J. and Alexander, N.M. (1985) J. Biol. Chem. 260, 15168–15171.
[18] Ehresman, B., Imbault, P. and Weil, I.H. (1973) Anal. Biochem. 54, 454–463.
[19] Worthington, D.J. and Rosemeyer, M.A. (1976) Eur. J. Biochem. 67, 231–238.
[20] Prongay, A.J., Engelke, D.R. and Williams Jr., C.H. (1989) J. Biol. Chem. 264, 2656–2664.
[21] Rietveld, P., Arscott, L.D., Berry, A., Scrutton, N.S., Deonarain, M.P., Perham, R.N. and Williams Jr., C.H. (1994) Biochemistry 33, 13888–13895.
[22] Davis, R.E. and Swain, C.G. (1960) J. Am. Chem. Soc. 82, 5949–5950.
[23] Mulrooney, S.B. (1997) Prot. Expr. Purif. 9, 372–379.
[24] Eftink, M.R. (1991) in: Methods of Biochemical Analysis. Fluorescence Techniques for Studying Protein Structure (Suelter, C.H., Ed.) vol. 35, pp. 127–205, John Wiley and Sons, New York.
[25] Becker, K. and Schirmer, R.H. (1995) Methods Enzymol. 251, 173–188.
[26] Daemen-Gubbels, C.R., Groeneveld, P.H., Groeneveld, A.B., van Kamp, G.J., Bronsveld, E. and Thijs, L.G. (1995) Crit. Care Med. 23, 1363–1370.
[27] Luo, D., Das, S. and Vincent, S.R. (1995) Eur. J. Pharmacol. 290, 247–251.
[28] Coleman, M.D. and Coleman, N.A. (1996) Drug Saf. 14, 394–405.
[29] Atamna, H., Krugliak, M., Shalmiev, G., Deharo, E., Pescarmona, G. and Ginsburg, H. (1996) Biochem. Pharmacol. 51, 693–700.
[30] Clark, G. (1981) Staining Procedures, 4th Edn., p. 173, Williams and Wilkins, Baltimore, MD.
[31] Schönleben-Janas, A., Kirsch, P., Mittl, P.R.E., Schirmer, R.H. and Krauth-Siegel, R.L. (1996) J. Med. Chem. 39, 1549–1554.
[32] Karplus, P.A., Pai, E.F. and Schulz, G.E. (1989) Eur. J. Biochem. 178, 693–703.
[33] Arscott, L.D., Veine, D.M. and Williams Jr., C.H. (1997) in: Flavins and Flavoproteins (Stevenson, K.J., Massey, V. and Williams Jr., C.H., Eds.) vol. 12, pp. 679–682, University of Calgary Press, Calgary.