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FAD analogues as prosthetic groups of human glutathione reductase

Properties of the modified enzyme species and comparisons with the active site structure

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1. Human glutathione reductase (NADPH + GSSG + $H^+ \rightleftharpoons NADP^+$ + 2 GSH) is a suitable enzyme for correlating spectroscopic properties and chemical reactivities of protein-bound FAD analogues with structural data. FAD, the prosthetic group of the enzyme, was replaced by FAD analogues, which were modified at the positions 8, 1, 2, 4, 5 and 6, respectively, of the isoalloxazine ring.

2. When compared with a value of 100% for native glutathione reductase, the specific activities of most enzyme species ranged from 40% to 17%, in the order of the prosthetic groups 8-mercapto-FAD > 8-azido-FAD = 8-F-FAD = 8-Cl-FAD > 4-thio-FAD = 1-deaza-FAD > 2-thio-FAD. The enzymic activities indicate a correct orientation of the bound analogues. The enzyme species containing 5-deaza-FAD and 6-OH-FAD, respectively, had no more glutathione reductase activity than the FAD-free apoenzyme. 5-Deaza-FAD · glutathione reductase was crystallized for X-ray diffraction analysis.

3. Detailed studies were focussed on position 8 of the flavin. 8-Cl-FAD · glutathione reductase and 8-F-FAD · glutathione reductase reacted only poorly with HS^- to give 8-mercapto-FAD · glutathione reductase, which suggests that the region around Val61 hinders the halogen anion from leaving the tetrahedral intermediate. Other experiments showed that position 8 is accessible to certain solvent-borne reagents. 8-Mercapto-FAD · glutathione reductase, for instance, reacted readily and stoichiometrically with the thiol reagent methylmethanethiosulfonate.

4. 8-Mercapto-FAD · glutathione reductase does not exhibit a long wavelength charge transfer absorption band upon reduction, as it is the case for the 2-electron-reduced FAD-containing enzyme. This behaviour indicates that the charge transfer interaction between flavin and the thiolate of Cys63 in the native enzyme is not *per se* essential for catalysis.

The absorption spectrum of the blue anionic 8-mercapto-FAD bound to glutathione reductase suggests that the protein concurs to the stabilization of a negative charge in the pyrimidine subnucleus. In light of the protein structure this effect is attributed to the dipole moment of α-helix 338 – 354 which starts out close to the N(1)/C(2)/O(2α) region of the flavin.

5. 1-Deaza-FAD binds as tightly as FAD to the apoenzyme. The resulting holoenzyme was found to be enzymically active but structurally unstable. In this respect 1-deaza-FAD · glutathione reductase mimics the properties of the enzyme species found in inborn glutathione reductase deficiency. The high specific activity of 1-deaza-FAD · glutathione reductase shows that the hydrogen bond between N(1) and Thr339 in the native enzyme is not essential for catalysis.

6. 2-Thio-FAD and 4-thio-FAD were used as probes in order to confirm the crystallographic result that the region around N(1)/O(2α)/N(3) and around O(4α) of the native enzyme are practically inaccessible to solvent-borne reagents. Reaction of 4-thio-FAD · glutathione reductase with a large excess of H_2O_2 very slowly led to a catalytically inactive protein which may contain covalently bound FAD.

In contrast to the native FAD enzyme, 2-electron-reduced 4-thio-FAD · glutathione reductase contains reduced flavin whereas the redox-active pair Cys58 – Cys63 is likely to be present in the disulfide form. As 4-thio-FAD is a catalytically competent prosthetic group, this means that the reducing equivalents can flow from NADPH via the two redox systems of the enzyme to GSSG even when the flavin analogue has a less negative redox potential than the subsequent dithiol/disulfide group.

7. 6-OH-FAD · Glutathione reductase showed no detectable reductase activity. The p*K* value of 7.2 ± 0.3 found for protein-bound 6-OH-FAD is the same as for free 6-OH-FAD. The interpretation that 6-OH-FAD binds poorly to apoglutathione reductase is consistent with the structure of the native enzyme. Three amino acid residues, namely Gly62, Cys63 and Lys66, are in van der Waals contact with C(6) and do not allow the introduction of a bulky OH-group at this position. The discrimination against 6-OH-FAD which occurs beside FAD *in vivo* might be the result of a selection process.

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Dedicated to Professor Reinhard Brossmer on the occasion of his 60th birthday.

Abbreviations. MMTS, methylmethanethiosulfonate; GR, glutathione reductase from human erythrocytes; BCNU, carmustine, *N,N'*-bis(2-chloroethyl)-*N*-nitrosourea; E, enzyme with the catalytic site being in the oxidized state; EH_2, enzyme after the uptake of two reducing equivalents.

Enzymes. Glutathione reductase [NAD(P)H] (EC 1.6.4.2); lipoamide dehydrogenase or NADH: lipoamide oxidoreductase (EC 1.6.4.3); *p*-hydroxybenzoate hydroxylase or 4-hydroxybenzoate 3-monooxygenase (EC 1.14.13.2).

Recent progress in our understanding of flavoenzyme catalysis has been achieved along two major lines. One approach, the use of FAD and FMN analogues with specific modifications in the flavin moiety, was intraduced in 1976 [1] and subsequently applied in the study of more than 20 enzymes by Massey and collaborators [2, 3]. Modified flavins can serve as probes for studying various properties of an active site such as the dielectricity of the flavin-binding pocket, the location and orientation of electric charges and dipoles in this pocket, the shielding of different flavin subfunctions from the solvent, hydrogen bonds between specific flavin positions and functional groups of the apoprotein, and the effect of the flavin redox potential on individual catalytic steps. The information arising from such studies relies largely on the interpretation of spectral modifications and perturbations which occur upon binding of the flavin analogues to the apoprotein, or upon the interaction of the flavin-modified enzyme with substrates or inhibitors.

In a second approach, the structures of the flavoenzymes *p*-hydroxybenzoate hydroxylase from *Pseudomonas fluorescens* [4] and glutathione reductase from human erythrocytes [5] were elucidated at 0.2 nm resolution so that, in these cases, the interaction of flavin and apoenzyme can be studied in atomic detail [6–8]. For glutathione reductase the stereochemistry of catalysis has been established as well [9]. Consequently correlations between spectral properties of protein-bound analogues on the one hand and an active centre structure of an enzyme on the other hand can be attempted. This is the main purpose of the work reported here.

In addition, FAD analogues are promising tools for analyzing and inhibiting the reactions catalyzed by glutathione reductase. The latter point is also of practical importance because inhibitors of this enzyme such as carmustine (BCNU) are used clinically in the chemotherapy of malignancies [10, 11] and experimentally in malaria research [12, 13].

Glutathione reductase has an M_r of 105000 [5, 14] and consists of two identical FAD-containing subunits connected by a disulfide bridge [15]. The enzyme catalyzes the reaction NADPH + GSSG + $H^+ \rightleftharpoons NADP^+$ + 2 GSH. *In vitro* [16] and probably also *in situ* [17], glutathione reductase can occur in two stable states, namely E and EH_2. In E, both flavin and the disulfide bridge Cys58–Cys63 are in the oxidized form (Fig. 1); in EH_2, produced according to the equation E + NADPH + $H^+ \rightarrow EH_2 + NADP^+$, flavin is in the (re)oxidized form whereas Cys58 and Cys63 are present as a dithiol [9, 16].

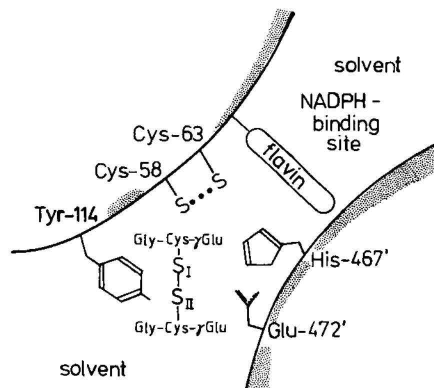

Fig. 1. *Sketch of the active site of glutathione reductase.* During catalysis [9, 16] reducing equivalents flow from NADPH via flavin and open the disulfide bridge Cys58–Cys63. Then the thiol group of Cys58, a strong nucleophile, attacks sulfur I of glutathione disulfide

MATERIALS AND METHODS

Methylmethanethiosulfonate (MMTS) and 2′-monophosphoadenosine 5′-diphosphoribose (2′-phosphoryl-ADP-ribose) were purchased from Sigma and $Na_2S \cdot 5\ H_2O$ was from Riedel de Haën. Iodoacetamide and iodoacetic acid (from Merck) were recrystallized from *n*-heptane. Absorption spectra and kinetic data were recorded on a Pye Unicam SP8-100 spectrophotometer or a Kontron spectrophotometer Uvikon 820.

The FAD analogues were prepared from the corresponding riboflavins using the FAD-synthesizing system from *Brevibacterium ammoniagenes* and purified according to Spencer et al. [18] or by high performance liquid chromatography as described by Wenz et al. [19]. 8-Cl-Riboflavin, 8-F-riboflavin, and 1-deazariboflavin were kind gifts from Professor V. Massey, 2-thioriboflavin [20], 4-thioriboflavin [21], riboflavin-4-*S*-oxide [21], 5-deazariboflavin [22], 6-hydroxyriboflavin [23], and 8-azidoriboflavin [24] were prepared according to published procedures. 8-Mercapto-FAD was obtained by incubating 8-Cl-FAD at pH 9 with 5 mM Na_2S overnight at 25 °C. Glutathione reductase from human erythrocytes was purified [25] and assayed [26] as previously described. The FAD-free apoenzyme (M_r of one subunit = 51600 [14]; $A_{280\,nm}^{1\,mM}$ = 50.0 [27]) was prepared by $(NH_4)_2SO_4$ precipitation at pH 3.0 [27, 28] and dissolved in 100 mM Tris/HCl, 10 mM EDTA, pH 8.7, at a concentration of 5–10 mg/ml (=0.1–0.2 mM). The Tris buffer was used because the apoenzyme is poorly soluble in neutral phosphate buffers. Reconstitution of the enzyme with an FAD analogue was achieved by adding 1 ml of the apoenzyme solution to 1.5–3 molar excess FAD analogue dissolved in 0.5 ml buffer A (=100 mM potassium phosphate, 200 mM KCl, 1 mM EDTA, pH 6.9). After 2–6 h at 4°C, the reaction mixture was passed through a Sephadex G-25 column (0.73 × 21 cm), equilibrated with buffer A, in order to remove excess FAD analogue and to change the buffer. When studying the effects of apoenzyme on the spectrum of an analogue we reconstituted the holoenzyme in a cuvette by adding 50 µl stock solution (containing 4–6 nmol apoGR in Tris buffer) to 5 nmol FAD analogue dissolved in 1 ml buffer A.

In the presence of O_2, 5 µM solutions of GR species containing 8-Cl-FAD, 8-mercapto-FAD and 2-thio-FAD, respectively, catalyzed the oxidation of < 5 nmol NADPH/min in a volume of 1 ml. This means that their NADPH-oxidase activity is not higher than that of native FAD·GR [29].

RESULTS

Reconstitution of active glutathione reductase species with FAD analogues

The method for preparing glutathione reductase from apoenzyme and FAD analogues was applicable to all analogues. Most resulting enzyme species were enzymatically active when tested in the standard assay (Table 1). Routinely, the reproducibility of the preparative procedures was checked by reconstituting FAD·GR (with 100% enzyme activity) from FAD and apoenzyme, and by preparing 5-deaza-FAD·GR

Table 1. *Properties of glutathione reductase reconstituted with FAD analogues*
The enzyme activity was determined under standard assay conditions [25, 26]. The ε-value of 10.5 for enzyme-bound FAD is based on an $A^{1\%}_{463\,nm}$ = 2.01 [26] and an M_r of 52400 [14]. Most authors, however, use an ε-value of 11.3 both for free and enzyme-bound FAD [16]. The activity of 4-sulfoxo-FAD·GR may be identical with that of 4-thio-FAD·GR since 4-sulfoxo-FAD is likely to be deoxygenated in the first catalytic cycle (Massey, V., personal communication). No changes when compared with flavin

FAD analogue	Mode of flavin substitution						λ_{max} (ε)		GSSG-Reductase activity
	1	2α	4α	5	6	8	free	bound to apoGR in buffer A, pH 6.9	
							nm (mM^{-1} cm^{-1})		%
FAD	N	O	O	N	H	CH_3	450 (11.3) [30] 375 (9.3)	463 (10.5) 377 (9.4)	100
1-Deaza-FAD	CH	„	„	„	„	„	535 (6.4) [31] 365 (3.4)	560 (6.4) 365 (5.8)	22
2-Thio-FAD	„	S	„	„	„	„	486 (20.0) [32] 316 (30.0)	504 (20.0) 324 (40.0)	17
4-Thio-FAD	„	„	S	„	„	„	492 (13.0) [21] 368 (9.5)	505 (14.6) 372 (12.9)	24
4-Sulfoxo-FAD	„	„	SO	„	„	„	535 (~12.5) 376 (~12.5)	486 (~12.5) 376 (~14.2)	32
5-Deaza-FAD	„	„	„	CH	„	„	399 (11.5) [33] 338 (10.9)	408 (13.2) 340 (14.0)	0
6-OH-FAD	„	„	„	„	OH	„	422 (19.6) [23]	422 (21.0)	0
8-Cl-FAD	„	„	„	„	„	Cl	448 (10.6) [34] 364 (8.9)	458 (12.0) 360 (10.4)	30
8-F-FAD	„	„	„	„	„	F	436 (11.0)[a] [35] 356 (7.3)	446 (11.0) 346 (9.4)	33
8-Mercapto-FAD	„	„	„	„	„	SH	530 (28.6) [36]	575 (26.5) 455 (11.3)	40
8-Azido-FAD	„	„	„	„	„	N_3	448 (22.0)[a] [37]	462 (~22.0)	33

[a] Values reported for riboflavin, not for FAD.

(with no detectable enzyme activity) from 5-deaza-FAD and apoenzyme. Both reconstituted FAD·GR and 5-deaza-FAD·GR could be crystallized according to [25, 26].

For spectroscopic studies about 5 μM GR in buffer A was used. With the exceptions of 1-deaza-FAD·GR and 6-OH-FAD·GR, a final stable state characterized by a constant spectrum was reached which indicates that the prosthetic group is tightly bound to the protein moiety in a unique fashion. The finding that the specific enzyme activity did not decrease with dilution (down to 50 ng/ml = 1 pmol/ml) confirmed the stability of the reconstituted enzyme species. The data for the enzyme analogues are presented in the following order: (a) activity of the reconstituted enzyme; (b) influence of the apoprotein moiety (in the oxidized state E) on the absorption spectrum (Table 1) and on the chemical reactivities of the FAD analogue; (c) spectral properties of the enzyme after reduction with its substrate NADPH. The evaluation of the results with respect to structure and function of glutathione reductase is left to the Discussion.

8-Cl-FAD·GR

When compared with native FAD·GR, 8-Cl-FAD·GR possesses ≈30% activity. The absorption spectra of 8-Cl-FAD and of FAD, respectively, change only slightly and in a similar way when they are bound to apoglutathione reductase. Consequently no modification of the flavin moiety occurs on binding of 8-Cl-FAD to the protein. This contrasts with the situation in the closely related enzyme lipoamide dehydrogenase where the 8-Cl group reacts with the −SH group of a cysteinyl residue giving rise to a thioether link between FAD analogue and protein [34]. The known reactivity of 8-Cl-FAD towards thiols [34, 38] suggested modifying 8-Cl-FAD·GR with Na_2S. However, even under drastic conditions (16 mM Na_2S, pH 10.1, 19 h reaction time, 4°C) only 19% of the spectral changes expected for the formation of an 8-mercapto-FAD enzyme (see below and Fig. 3) were observed. Under standard conditions (8 mM Na_2S, pH 7.1, 2 h reaction time at 25°C) only 8% of the expected species was formed. Using comparable conditions, the half-time for the conversion of free 8-Cl-FAD to 8-mercapto-FAD was found to be 19 min which agrees well with the value of 20 min determined by Schopfer et al. [36].

Reduction of 8-Cl-FAD·GR with NADPH. Addition of NADPH to 8-Cl-FAD·GR in stoichiometric amounts causes no spectral changes, either because the spectra of E and EH_2 are indistinguishable or more likely because EH_2 is rapidly reoxidized by dioxygen. Upon addition of excess NADPH, several species were formed sequentially (Fig. 2). Eventually after 120 min, a stable species with a characteristic band at 410 nm and little absorption beyond 520 nm appeared. In analogy to a similar spectrum observed with native yeast glutathione reductase this species might represent a flavin semiquinone [16].

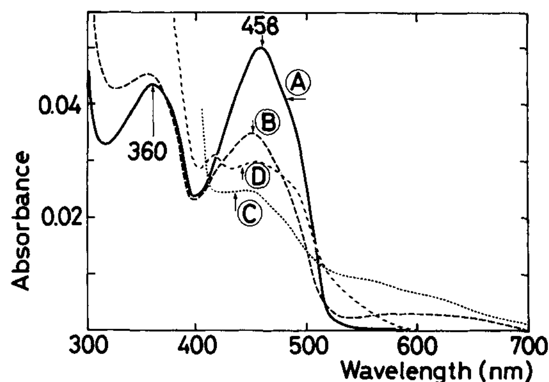

Fig. 2. *Reduction of 8-Cl-FAD·GR with NADPH.* (A) spectrum of 8-Cl-FAD·GR (4.2 μM in buffer A, pH 6.9); (B) spectrum after addition of a 10-fold excess of NADPH; (C) spectrum after addition of 120 equivalents NADPH. The band around 600 nm of spectrum B reflects the formation of a charge transfer complex either between EH_2 and NADPH or, more probably, between EH_4 and $NADP^+$ [41]. Before spectrum D develops, flavin is progressively reduced while the long wavelength absorption reaches a steady state level. (D) Final spectrum obtained from C after 2 h in the presence of O_2 (see text for further comments)

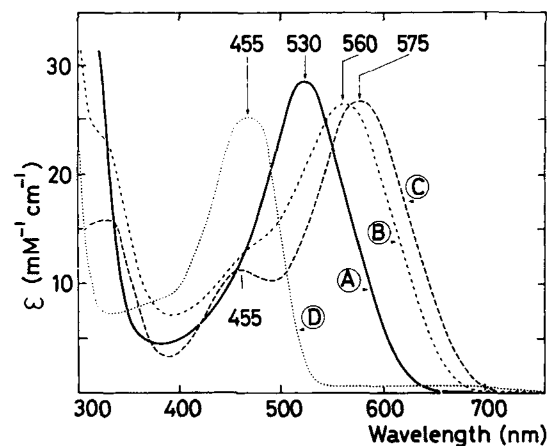

Fig. 3. *Binding of 8-mercapto-FAD to apoglutathione reductase and the reaction of the reconstituted enzyme with methylmethanethiosulfonate.* 5 μM 8-mercapto-FAD in buffer A, pH 6.9 (A) was titrated with apoGR at 25 °C. The spectrum was recorded immediately after titration (B) and 12 h later (C). Spectrum C represents 8-mercapto-FAD·GR. Spectrum D resulted from reacting 8-mercapto-FAD·GR with one equivalent MMTS

8-Fluoro-FAD·GR

In context with the unexpectedly low reactivity of 8-Cl-FAD·GR with sulfide, 8-F-FAD was investigated which is more reactive than 8-Cl-FAD towards weak nucleophiles [35]. Binding of 8-F-FAD to apoglutathione reductase leads but to small shifts in the absorption spectrum. The modified enzyme possesses ≈ 33% of the reductase activity of the native enzyme (Table 1). The conversion of 8-F-FAD·GR to 8-mercapto-FAD·GR though also very slow, proceeded faster than in the case of 8-Cl-FAD enzyme. Thus addition of 2 × 11 mM HS^- at a final pH of 8.8 led to about 50% conversion within 20 h. In comparison, free 8-F-FAD reacted with 15 mM HS^- within 10 min to 70% formation of the 8-mercapto analogue, incubation for 10 h at 25 °C did not lead to further changes. A complex kinetic behaviour of HS^- has also been observed with other flavoproteins reconstituted with 8-halogeno flavins [36].

8-Mercapto-FAD·GR

Upon incubation of 8-mercapto-FAD with 1.2 equivalents apoenzyme the 530-nm band of the chromophore shifted to 560 nm (spectrum B in Fig. 3) and then within 12 h to 575 nm (spectrum C). The spectral changes probably reflect a very slow protein isomerisation subsequent to a primary binding step. All studies were done with the final stable form of 8-mercapto-FAD·GR which was 40% active when compared with the native enzyme (Table 1).

8-Mercapto-FAD·GR reacts readily and stoichiometrically with methylmethanethiosulfonate, a reagent which is used for probing the accessibility and reactivity of mercaptide groups [39]. The reaction occurs isosbestically; the spectrum of the product (D in Fig. 3) is characteristic of 8-S-alkylated flavins [38], which indicates that an 8(-$SSCH_3$)-flavindisulfide is formed. As shown in Table 2, the reaction between 8-mercapto-FAD·GR and methylmethanethiosulfonate is three times slower in the presence of 2′phosphoryl-ADP-ribose, which binds in the NADPH pocket of the enzyme [40]. After addition of 10 mM 2-mercaptoethanol the original spectrum of the 8-mercapto-FAD·enzyme (C in Fig. 3) is restored. 8-Mercapto-FAD·GR reacts irreversibly with iodoacetamide and iodoacetate, respectively (Table 2). Both products, probably 8-carboxamidomethyl-S-FAD·GR and 8-carboxymethyl-S-FAD·GR, possess typical spectra of 8-S-alkylated flavins [38] which are closely similar to that obtained with methylmethanethiosulfonate.

Spectral properties of the EH_2 state. The addition of one equivalent of NADPH to 8-mercapto-FAD·GR leads to minor but distinct spectral changes: a slight hypsochromic shift of the long wavelength maximum to 568 nm and a decrease of the absorbances at 568 nm and at 444 nm by 10% (spectrum B in Fig. 4). An absorption increase at 340 nm which would indicate the presence of unreacted NADPH was not observed. In conclusion, spectrum B in Fig. 4 represents 8-mercapto-FAD·EH_2. This spectrum does not show the charge transfer band which is characteristic of FAD·EH_2 in the native enzyme [16, 41, 42].

Reduction of the flavin, which can be followed by the disappearance of the absorbance maximum at 568 nm, was only achieved by adding NADPH in large excess to 8-mercapto-FAD·EH_2 (spectrum C in Fig. 4). The molecular species represented by spectrum C is probably 8-mercapto-$FADH_2$·EH_2 (EH_4). Within 12 h at 4 °C, the high absorbance at 340 nm disappeared which indicates a spontaneous or more likely, enzyme-catalyzed oxidation of NADPH by dioxygen. As shown in spectrum D, a new molecular species with a maximum at 606 nm was obtained. This species is probably the complex of reoxidised 8-mercapto-FAD·E and $NADP^+$.

Complementary studies such as the anaerobic titration of 8-mercapto-FAD·GR with dithionite, and the titration of the oxidized enzyme with $NADP^+$ will help to obtain more insight into the molecular species represented in Fig. 4.

1-Deaza-FAD·GR

Binding of 1-deaza-FAD to apoGR leads to a shift of the long wavelength band of the chromophore from 535 nm [31]

Table 2. *Rates of reaction of 8-mercapto-FAD·GR and of free 8-mercaptoflavins with sulfhydryl reagents*
MMTS, methylmethanethiosulfonate

Flavin	Reagent	$t_{1/2}$	Reaction rate
		min	M^{-1} min^{-1}
8-Mercapto-FAD·GR (5 μM)	MMTS (5 μM)	1.0	1.4×10^5
8-Mercapto-FAD·GR (5 μM), in the presence of 0.5 mM 2′-phosphoryl-ADP-ribose	MMTS (5 μM)	3.4	4.1×10^4
8-Mercaptolumiflavin (1 μM)	MMTS (1 μM)	0.5	1.4×10^6
8-Mercapto-FAD·GR (1.5 μM)	iodoacetamide (10 mM)	30	2.3
8-Mercapto-FAD·GR (1.5 μM)	iodoacetic acid (10 mM)	56	1.3
8-Mercapto-FAD	iodoacetamide (12.5 mM)		46.3 [36]
8-Mercapto-FAD	iodoacetic acid (12.5 mM)		9.8 [36]

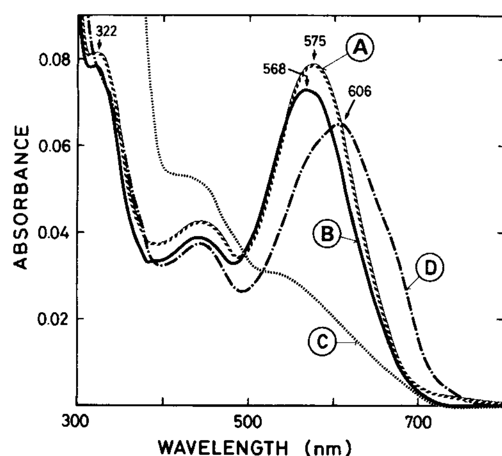

Fig. 4. *Reaction of 8-mercapto-FAD·GR with NADPH.* 8-Mercapto-FAD·GR in buffer A, pH 6.9 at 25°C, (A) was mixed with one equivalent NADPH (B) and 20 equivalents NADPH (C). Spectrum C was recorded immediately and D, the final spectrum, after 12 h

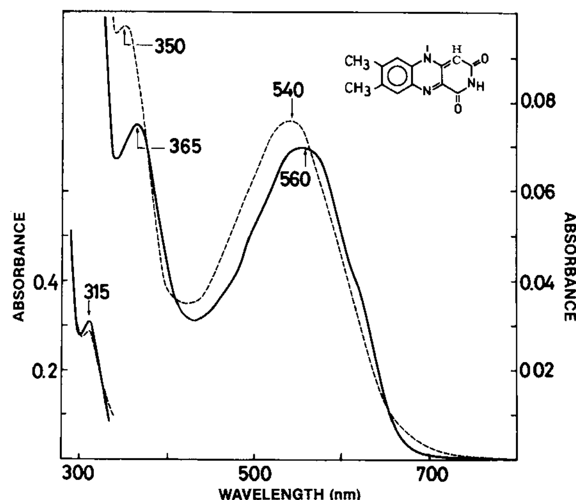

Fig. 5. *Anaerobic reduction of 1-deaza-FAD·GR reductase with NADPH.* Curve (——) represents the absorption spectrum of 11 μM 1-deaza-FAD·GR in 100 mM Tris/HCl, 10 mM EDTA, pH 8.7 at 2°C. Anaerobic addition of NADPH to a final concentration of 14 μM led to formation of spectrum (– – – –). Further anaerobic addition of NADPH (500 μM) did not cause further changes at wavelengths > 400 nm

to 560 nm, and to a 70% increase of the band at 365 nm (Fig. 5). 1-Deaza-FAD·GR is stable in solution at 2°C, but develops turbidity indicating denaturation at room temperature. The dissociation constant for the complex between 1-deaza-FAD and apoGR was ≲ 0.17 μM when determined according to Staal et al. [43] who found an apparent $K_{diss} = K_m$ of 0.55 μM for the native FAD·GR complex. This comparison indicates that the precipitating species is indeed 1-deaza-FAD·GR and not the apoenzyme (which is also insoluble in neutral phosphate buffers). Anaerobic addition of NADPH gives rise to a species analogous to state EH_2 of the native enzyme. Evidence for this is that NADPH is oxidized as seen by the decrease in absorbance at 340 nm while 1-deazaflavin is not reduced (Fig. 5). The shift of λ_{max} from 560 nm to 540 nm and the lack of a long wavelength absorption band correspond to properties of the EH_2-state of 1-deaza-FAD·lipoamide dehydrogenase (Massey, V., personal communication). 1-Deaza-FAD·GR was found to have 22% activity when compared with FAD·GR (Table 1).

2-Thio-FAD·GR

The modified enzyme containing a sulfur instead of an oxygen in position 2α of FAD, possesses 17% of the activity of normal FAD·GR (Table 1). This activity does not result from a possible hydrolysis of 2-thio-FAD as the value of 17% remains constant independently of the time of incubation.

The absorption spectrum of 2-thio-FAD·GR is quite different from the spectrum of free 2-thio-FAD (Table 1): when 2-thio-FAD is bound to apoglutathione reductase, the absorbance maxima shift from 486 nm to 504 nm and from 316 nm to 324 nm, respectively. This red shift has also been observed upon binding of 2-thio-FAD to other apoenzymes [32].

In analogy to the experiments with 8-mercapto-FAD·GR (see above), 2-thio-FAD·GR was incubated with MMTS in order to test the accessibility of the region N(1)/C(2)/N(3) of the bound flavin. Free 2-thio-FAD and accessible enzyme-bound 2-thio-FAD react readily with MMTS; the products, free and enzyme-bound 2(-$SSCH_3$)-FAD, respectively, exhibit characteristic absorption spectra [32]. As judged from the lack of spectral changes, 2-thio-FAD·GR did not react with MMTS, even not under drastic experimental conditions (up to 10 mM MMTS, 24 h incubation time, pH range 6–9, temperature range 4–25°C).

4-Thio-FAD·GR

Reconstitution of apoGR with 4-thio-FAD leads to a shift of the absorption maximum from 486 nm to 505 nm. This

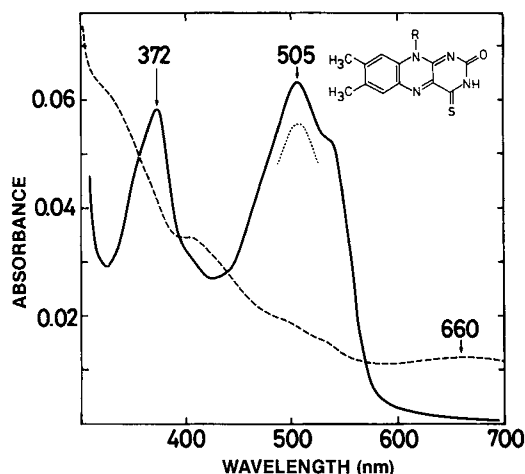

Fig. 6. *Anaerobic reduction of 4-thio-FAD·GR with NADPH.* 4.3 μM 4-thio-FAD·GR in buffer A, pH 6.9 at 2 °C (——) was reduced by 8 μM NADPH under anaerobic conditions (– – – –). Upon admission to air this species decayed over a period of several hours to yield 90% of the starting 4-thio-FAD·enzyme (· · · · · ·). Spectrum (– – – –) probably represents a complex between 4-thio-FAD·GR containing reduced flavin and $NADP^+$

enzyme species shows 24% catalytic activity when compared with native FAD·GR (Table 1).

Incubation of 4-thio-FAD·GR (4.5 μM) with a 50-fold excess of MMTS for 1 h had no effect on the absorption spectrum. In contrast, free 4-thioflavin reacts readily with this reagent or with H_2O_2. H_2O_2 did also attack 4-thio-FAD·GR, but only very slowly and when present in large excess. The reaction led to the disappearance of the typical 4-thioflavin spectrum and to the isosbestic formation of a molecular species with absorption maxima at 452 nm and 364 nm. This enzyme species – which has no detectable GSSG reductase activity – possibly contains FAD covalently linked to a side chain of the protein. Lys66 with its ε-NH_2 group at a distance of 0.3 nm to position O(4α) in the native enzyme would be a good candidate; the long side-chain of this residue lines the flavin around C(6)/N(5)/O(4α) [6]. In order to test our hypothesis, peptide mapping of the modified glutathione reductase species is in progress.

Reduction of the flavin in 4-thio-FAD·GR was achieved rapidly and quantitatively with less than 2 molar excess of NADPH under anaerobic conditions, which means that reduced 4-thio-FAD·GR – unlike EH_2 of the native enzyme – contains reduced flavin and probably a closed disulfide bridge at the active site. This interpretation is consistent with the high redox potential of free 4-thio-$FADH_2$/4-thio-FAD (–55 mV [21]).

The spectrum of reduced 4-thio-FAD·GR (Fig. 6) does not show the characteristic, resolved band of neutral reduced 4-thioflavins [21], but a nonresolved band around 400 nm. This might suggest that the reduced 4-thio-FAD in GR is in the anionic state. Whereas free 2e-reduced 4-thioflavins are rapidly reoxidized by atmospheric oxygen, this process occurs slowly in reduced 4-thio-FAD·GR. Only after 4 h at room temperature the oxidized species was regained with 90% yield. The detailed mechanism of the reaction between reduced 4-thio-FAD·GR and O_2 – which might involve a disulfide intermediate formed between apoprotein and chromophore – remains to be studied.

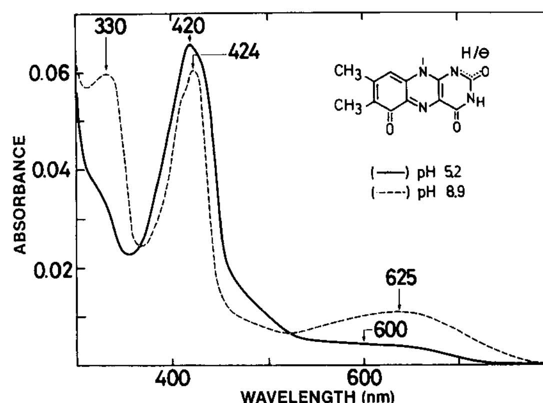

Fig. 7. *pH-dependence of the absorption spectrum of 6-OH-FAD·GR.* 3.1 μM 6-OH-FAD·GR in buffer A, pH 6.9 was titrated with 1 M acetic acid or 1 M Tris base at 2 °C. The spectra at the pH values of 5.2 (——) and 8.9 (– – – –) are shown. The estimated p*K* for the spectral changes at 330 nm and 680 nm is 7.2 ± 0.3

6-OH-FAD·GR

In contrast to most other GR species tested, 6-OH-FAD·GR has no detectable reductase activity (Table 1). Addition of equimolar concentrations of apoGR to 6-OH-FAD had no conspicuous effect on the spectrum (Table 1, Fig. 7). A pH titration of 6-OH-FAD·GR yielded an estimated p*K* of 7.2 ± 0.3 based on changes occurring at 330 nm and 680 nm. This might indicate, that the protein does not affect the p*K* of 6-OH-flavin [23], or, alternatively, that the 6-OH-flavin moiety of this FAD analogue does not interact strongly with functional groups of the apoprotein. The lack of enzyme activity and the dissociation of the chromophore upon ultrafiltration of 6-OH-FAD·GR are consistent with poor binding of 6-OH-FAD to apoGR. In contrast to the spectra of free 6-OH-flavins a long wavelength absorption was observed with 6-OH-FAD·GR at pH < p*K* (Fig. 7). Such a band is found with neutral 6-OH-flavins which are alkylated at position N(1) [44] and might indicate, that in 6-OH-FAD·GR, if protonation occurs, it is at position N(1), and not at O(6α).

DISCUSSION

Human glutathione reductase is well-suited for correlating flavoenzyme chemistry with structural data for the following reasons: (a) the contacts between apoenzyme and FAD are known [6], (b) preliminary X-ray analyses comparing apoenzyme [6] and 5-deaza-FAD·GR (Pai, E. F., personal communication) with FAD·GR have shown that the apoenzyme does not undergo major conformational changes when FAD or the FAD analogue is bound, (c) glutathione reductase is a member of an enzyme family which includes, for instance, lipoamide dehydrogenase [45] and mercuric ion reductase [46]. Thus not only analogues of the prosthetic group but also homologues of the apoenzyme are available for studying the interactions between flavin and apoprotein in a pinpointed fashion.

Interactions of specific functional groups of flavin analogues with apoglutathione reductase

Each protein-bound flavin analogue has specific environmental requirements which can only partially be fulfilled by

Table 3. *Protein atoms around specific positions of the isoalloxazine ring within 0.45 nm*
Taken from [5] and Schulz, G. E. (personal communication). *, Number of additional atoms in the amino acid residue which are closer than 0.45 nm to this flavin position. The accuracy of the data is ± 0.05 nm

Flavin position	Amino acid	Closest atom	Distance	Flavin position	Amino acid	Closest atom	Distance
			nm				nm
N1	Cys63	S_γ	0.37	O2α	**Leu338	C, C_α	0.40
	*Leu338	C_α	0.40		**Thr339	N	0.31
	**Thr339	N	0.34		**Pro340	C_δ	0.28
					***His467	O	0.35
O4α	*Cys63	C_α	0.39	N5	*Gly62	C	0.41
	*Lys66	N_ζ, C_ε	0.30		**Cys63	N, C_α	0.35
	Phe372	$C_{\varepsilon2}$, $C_{\delta2}$	0.41		**Lys66	C_ε,	0.30
	*His467	O	0.31		*Tyr197	O_η	0.42
	**Pro468	C_δ	0.30		Glu201	$O_{\varepsilon2}$	0.44
C6	*Gly62	C, C_α	0.35	C8	Thr57	$C_{\gamma2}$	0.44
	*Cys63	N	0.37		Val61	O	0.42
	**Lys66	C_ε	0.38		Gly62	C_α	0.39
	Tyr197	O_η	0.44		Tyr197	O_η	0.44
	Glu201	$O_{\varepsilon2}$	0.44		Ile198	C_δ	0.39
C8α	Thr57	$C_{\gamma2}$	0.40				
	Val61	O	0.39				
	Ser177	O_γ	0.42				
	Ile198	C_δ	0.36				
	*Arg291	N_η	0.40				

the apoenzyme. Examples illustrating the effects of the apoprotein are given here for the flavin positions 8, 1, 2, 4, and 6, respectively.

Substitutions at position 8. In native FAD-containing glutathione reductase, atoms contributed by Val61, Gly62 and Ile198 are in van der Waals contact with C(8) and C(8α) (Table 3). In addition, the 8-methyl group is at a distance of 0.4 nm to N_η of Arg291. These steric constraints are presumably the reason why in the 8-Cl-FAD·enzyme or 8-F-FAD·enzyme a nucleophilic substitution by HS^- at C(8) is hindered whereas reactions of the 8-mercapto-FAD·enzyme are fast. The substitution reaction at C(8) requires the formation of a tetrahedral intermediate which must be formed by nucleophilic attack perpendicular to the flavin plane. Although this attack seems possible, the halide ion is prevented from leaving by the very hydrophobic environment of position 8 in native FAD·GR (Table 3). In contrast, the access to position 8 appears to be unhindered in the flavin plane: the sulfur of 8-mercapto-FAD·GR reacts, for instance, very fast and stoichiometrically with MMTS to give the 8($-SSCH_3$) compound. In this case the attack occurs at position 8α and does not involve the transient formation of a tetracoordinated species. As one equivalent of MMTS is sufficient for complete conversion, interference by the modification of the eight sulfhydryl groups of the protein is excluded. The situation might be different in EH_2 where an unusually strong nucleophile, the thiol of Cys58, exists.

Positions 1/2α/3. 8-Mercapto-FAD appears to possess the *p*-quinoid structure when bound to glutathione reductase (Fig. 3) which implies that a negative charge is fixed in the region N(1)/O(2α) of flavin. This leads to the question of how this charge could be compensated. As shown by X-ray crystallography, a protein-bound cation such as the guanidinium group of an Arg residue is not close by; neither can solvent-borne cations contact the flavin here since the pyrimidine moiety of the isoalloxazine ring is buried inaccessibly within the protein structure [6–8]. In the native enzyme the N(1)/O(2α) locus is fixed to the amide group of Thr339 by a weak H-bond; N(3) forms a short and therefore strong H-bridge to the carbonyl group of His467. As shown in this report, reaction between enzyme-bound 2-thio-FAD and methylmethanethiosulfonate is impossible. This indicates that even the bulky sulfur atom replacing O(2α) does not disrupt the tight structure around the pyrimidine subnucleus.

Most likely, the negative charge at the N(1)/O(2α) locus in 8-mercapto-FAD·GR is stabilized by α-helix 338–354 which starts out close to this locus [6]. The electrical field of the helix-dipole [47] corresponds to half a positive charge at a distance of 0.3–0.4 nm from the N(1)/O(2α) locus. The ion pair His467–Glu472 (Fig. 1) with the positive charge facing O(2α) may be also of some importance [6]. We have expanded on this point because the stabilization of a negative charge at N(1)/O(2α) is expected to play a role for the catalytic mechanism of native glutathione reductase (see below). In conclusion, properly oriented dipoles in the interior of proteins – e.g. the salt bridges and the α-helices around the flavin in the interior of glutathione reductase – appear to be more elegant means for creating electric fields at specific loci than charged amino acid side chains.

Positions 4α and 5. The lack of reactivity of 4-thio-FAD ·GR towards methylmethanethiosulfonate confirms the crystallographic result that position 4α is tightly surrounded by atoms of the apoprotein [6] (Table 3). Nevertheless the protein scaffolding seems not to be absolutely rigid here since both sulfur and the even bulkier sulfoxo group can replace O(4α) of native FAD (Table 1) and since, although slowly, H_2O_2 reacts with 4-thio-FAD·GR at this position.

The fact that the flavin of 4-thio-FAD·GR – a catalytic competent enzyme species – can be reduced easily with NADPH raises the question as to the structure of the pros-

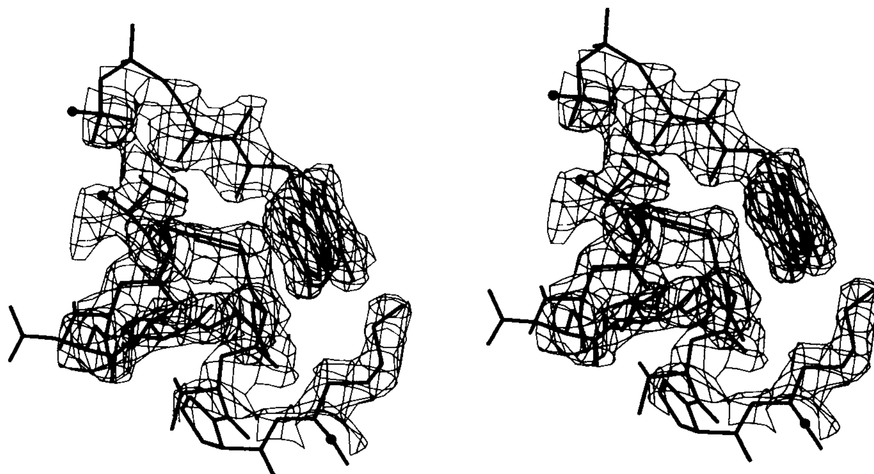

Fig. 8. *The active site structure at the si-face of flavin.* The electron density is represented in a chicken-wire-like structure and the molecular model in bold lines. Flavin-ribityl-diphosphate reaches from the centre at the right to the upper left hand side. The redox-active disulfide bridge Cys58 – Cys63, marked by a double line, stands out in the centre of the stereo picture. The polypeptide segment Thr57 – Lys66 extends from the upper left to the lower right hand side. It is easier to follow upstream from Lys66 (whose carboxyl C is indicated by a dot) via Pro65-Val64-Cys63-Gly62(below the disulfide bridge)-Val61-Asn60(whose side chain sticks out at the left hand side)-Val59 and Cys58 to Thr57 whose amido N is marked with a dot (drawn after [6])

thetic group after reduction. Crystallographic studies of native FAD·GR strongly suggest that N(5) is so tightly and rigidly surrounded by atoms of the apoprotein that a hydrogen atom cannot be accomodated here [6, 9] (Table 3). Consequently the structural data do not support a mechanism involving a direct hydride transfer from NADPH to the flavin N(5) position. In order to clarify the steric requirements for the accomodation of an H-atom at N(5) it is planned to analyze the three-dimensional structure of 2-electron-reduced 4-thio-FAD·GR and to compare it with reduced FAD·GR [6, 9].

Sulfite is often used as a model compound for successfully demonstrating a nucleophilic addition at N(5) [48]. For steric reasons [6] no sulfite adduct is formed in GR [48]. As mentioned, the protein structure is very tight around N(5)/O(4α); in particular it is the long side chain of Lys66 which lines the flavin in this region [5, 6] (Fig. 8).

Position 6α. The fact that 6-OH-FAD binds but weakly to glutathione reductase and that the reconstituted protein has no enzymic activity might be a consequence of natural selection since both FAD and 6-OH-FAD occur in mammalian cells [50]. Three amino-acid residues at the active site, namely Gly62, Cys63 and Lys66, contribute atoms which are in van der Waals contact with the methine group at position 6 and do not allow the presence of an extra oxygen here.

On the catalytic mechanism of glutathione reductase

Stabilization of a negative charge in the N(1)/O(2α) region as a possible prerequisite of enzyme activity. The interactions of apoglutathione reductase with FAD restrict the versatile chemistry of the isoalloxazine nucleus to a specific reaction pathway. The following hypothesis with respect to the nature of these interactions has been put forward [1, 48, 49]. A positive charge of the apoprotein is located close to the flavin N(1) position exerting here an inductive effect. This, in turn, catalyzes nucleophilic additions – e.g. in the form of reducing equivalents – to the flavin N(5)/C(4α) locus. The evidence that a negative charge can be stabilized in the pyrimidine portion of the flavin is as follows. (a) The structural analysis of glutathione reductase suggests a candidate for compensating this charge, namely the dipole field of helix 338 – 354 which starts out close to N(1)/O(2α) of the prosthetic group [6]. (b) The radical product which is formed by incubating 2-electron-reduced glutathione reductase (EH_2) with two equivalents of $NADP^+$ has been interpreted as a semiquinone anion [16]. According to Hemmerich [51], this radical anion requires the stabilization of a negative charge at position N(1)/O(2α) of flavin. (c) GR binds 8-mercapto-FAD predominantly in the blue form absorbing at 576 nm and showing the distinctive three-banded spectrum of the *p*-quinoid-bridged flavin [48] (Fig. 3). The *p*-quinoid form of 8-mercapto-FAD carries a negative charge at N(1)/O(2α) which must be compensated by the apoprotein. (d) The potential of stabilizing a negative charge close to N(1) would be impaired if N(1) were acceptor of a strong H-bond. The hypothesis that the H-bridge between N(1) of flavin and Thr338 is weak and not essential for catalysis (cf. [52]) is corroborated by the finding that 1-deaza-FAD which cannot form this bond at all, is a catalytically competent prosthetic group (Table 1).

The charge transfer complex in the 2-electron-reduced intermediate (EH_2). In the course of the reaction catalyzed by glutathione reductase, electrons flow from NADPH via flavin to the redox active protein disulfide and thence to the substrate GSSG (Fig. 1) [9, 16]. In the absence of GSSG, the 2-electron-reduced enzyme forms a stable intermediate (EH_2) characterized by an additional long wave absorption band and a reddish colour, whereas the enzyme in the oxidized state (E) is bright yellow. The interpretation that the different colour of EH_2 is caused by a charge transfer complex [41, 42] – (re)oxidized flavin being the acceptor and the thiolate of Cys63 the donor [5, 16] – is supported by our results. When 8-mercapto-FAD serves as a prosthetic group the spectrum of the enzyme does not show the charge transfer band; apparently the 8-mercapto-flavin with its delocalized negative charge cannot play the role of the acceptor. Since 8-mercapto-FAD·GR is catalytically active, an interpretation for the charge transfer band in native EH_2 can be put forward. The charge transfer absorption reflects the formation of a complex between two molecular partners having the correct reciprocal

orientation and redox potentials required for such a transition. This complex, however, may not be mandatory for catalysis and its existence might not imply that electrons are passed on via such an interaction. Results with a derivative of the closely related enzyme lipoamide dehydrogenase also suggest that the charge transfer complex *per se* is not an obligatory intermediate (see [53] for a detailed discussion).

The redox-reactive groups of the enzyme and the relative magnitude of their redox potentials. In most GR species containing an FAD analogue [54], the flavin has a lower redox potential than the Cys58—Cys63 pair so that the EH_2 state predominantly contains oxidized flavin and a dithiol at the active site. A notable exception is 2-electron-reduced 4-thio-FAD·GR, which probably represents an equilibrium mixture of molecular species with flavin being predominantly reduced and the sulfurs of Cys58—Cys63 being mainly in the disulfide form. The fact that the 4-thio-FAD·enzyme is 24% active when compared with native FAD·enzyme indicates that the flow of reducing equivalents is hardly impaired although there may be an energy barrier between 4-thioflavin and the active site disulfide.

Pharmacological aspects

BCNU, a clinically used cytostatic agent, is an inhibitor of glutathione reductase *in vivo* [55]. As GR protects tumour cells [11] and malaria parasites [13] against the oxidant stress exerted by macrophages, flavin analogues should be also studied as potential inhibitors of the enzyme [56]. Of special interest — but probably too toxic for clinical use — would be agents which turn GR into an oxidase [57, 58], that is, which make GR change sides in processes of oxidant stress versus antioxidant capacity. More promising are flavin analogues which destabilize glutathione reductase. A point in case is 1-deaza-FAD·GR which is catalytically active (Table 1) but structurally less stable than FAD·GR. In this respect 1-deaza-FAD·GR resembles the glutathione reductase species of inborn GR-deficiency [59]. In this condition all cells except mature red blood cells and eye lens fibres maintain a sufficient level of GR activity by increased *de novo* synthesis. GR-deficient erythrocytes are probably less suitable to serve as host cells for malaria parasites [56].

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