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Steady-State and Laser Flash Induced Photoreduction of Yeast Glutathione Reductase by 5-Deazariboflavin and by a Viologen Analogue: Stabilization of Flavin Adenine Dinucleotide Semiquinone Species by Complexation†

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ABSTRACT: Steady-state and laser flash photolysis techniques have been used to examine the photoreduction of yeast glutathione reductase by the one-electron reduction products of 5-deazariboflavin and the viologen analogue 1,1′-propylene-2,2′-bipyridyl. Steady-state photoreduction of the enzyme with the viologen generates the two-electron-reduced form, whereas photoreduction with deazaflavin generates the anion semiquinone. Flash photolysis indicates that the product of viologen radical reduction is also a semiquinone, suggesting that this species is rapidly further reduced by viologen in the steady-state experiment to form the EH_2 enzyme. This reduction is apparently inhibited when deazaflavin is the photoreductant, perhaps due to complexation of the anion semiquinone with deazaflavin. Steady-state experiments demonstrate that complexation of the anion semiquinone with $NADP^+$ also inhibits further reduction. Both one-electron reduction reactions of oxidized glutathione reductase proceed at close to diffusion-controlled rates (second-order rate constants = 10^8–10^9 M^{-1} s^{-1}), despite the relatively buried nature of the FAD cofactor. Addition of $NADP^+$ and oxidized glutathione produced no effects on the kinetics of the initial entry of the electron into the enzyme. No kinetic evidence of intramolecular electron transfer involving the FAD and the protein disulfide was obtained during or subsequent to the initial one-electron reduction process. Thus, if this reaction occurs in the semiquinone, it must be quite rapid ($k > 8000$ s^{-1}).

Glutathione reductase (GR) catalyzes the reduction of the disulfide bond of oxidized glutathione (GSSG), using NADPH as the source of reducing equivalents and an FAD cofactor and a protein disulfide as intermediate electron carriers (Williams, 1976). The enzyme isolated from human erythrocytes has been extensively characterized, and a refined X-ray structure at 1.54-Å resolution has been reported (Karplus & Schultz, 1987a). It is a dimer (total MW = 105 000) consisting of two identical subunits, each containing one FAD molecule (Krauth-Siegel et al., 1982). In the first part of the catalytic reaction, the enzyme is reduced to its stable EH_2 form by NADPH, from which $NADP^+$ dissociates. This species has an open disulfide (Cys-58–Cys-63 in the human enzyme; Pai & Schulz, 1983) resulting from electron transfer via the flavin, and spectroscopic data indicate the existence of a charge-transfer complex between a thiolate anion (the proximal

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Cys-63) and the oxidized flavin ring of the FAD cofactor (Kosower, 1966; Massey & Ghisla, 1974). In the absence of GSSG, the EH_2 species can bind $NADP^+$ and can slowly convert into a catalytically unimportant form tentatively identified as a complex of the FAD anionic semiquinone and $NADP^+$ (Bulger & Brandt, 1971; Williams, 1976). In the second stage of catalysis, GSSG binds to the EH_2 species and forms a mixed disulfide with Cys-58 (Pai & Schulz, 1983). This ultimately results in the formation of 2 mol of GSH and the regeneration of the oxidized enzyme.

In the present study, we have used one-electron reductants, specifically, 5-deazariboflavin semiquinone (5-dRfH$^\bullet$), and a reduced viologen analogue, 1,1′-propylene-2,2′-bipyridilium radical ($PDQ^{\bullet+}$), to reduce GR in both steady-state and transient kinetic experiments. The goals of this work were 3-fold: (a) to assess the accessibility of the FAD cofactor to nonphysiological exogenous electron donors, in view of the X-ray structure which indicates that the isoalloxazine ring is located in the center of each of the subunits of the enzyme and is not highly solvent exposed; (b) to determine the kinetic stability of the one-electron-reduced enzyme species; (c) to attempt to directly observe in a time-resolved experiment intramolecular electron transfer between the flavin and disulfide moieties in the enzyme.

As will be documented below, one electron can be introduced into the FAD cofactor from $PDQ^{\bullet+}$, and 5-dRfH$^\bullet$ at close to diffusion-controlled rates. This apparently occurs at a site that is removed from the substrate and cofactor binding areas and may be located in a region of the protein where the dimethylbenzene ring of the flavin is partly solvent accessible (Karplus & Schulz, 1987a,b). The resulting semiquinone species is stabilized against further reduction by complexing with $NADP^+$, and probably also with 5-deazariboflavin. Intramolecular electron-transfer events could not be observed, which suggests that these are quite rapid ($k > 8000\ s^{-1}$).

Materials and Methods

Yeast glutathione reductase was purified according to the method of Carlberg and Mannervik (1977). An extinction coefficient of $11.3 \times 10^3\ M^{-1}\ cm^{-1}$ at 463 nm was used to determine the concentration of oxidized protein. The diquat analogue 1,1′-propylene-2,2′-bipyridylium dibromide (PDQ^{2+}) was synthesized by using the procedure of Homer and Tomlinson (1980).

Phototitrations were performed by using 3-mL spectrophotometer cells equipped with a tapered neck and sealed with a rubber septum. Buffered solutions were made anaerobic by bubbling with argon for at least 90 min before the addition of aliquots of a concentrated enzyme stock. The buffer used for steady-state phototitrations was 25 mM Tris-HCl, pH 8.0, containing 1 mM EDTA and 10–15 μM 5-deazariboflavin (5-dRf). In some experiments 10 μM PDQ^{2+} and/or 2 mM $NADP^+$ was added. Visible spectra were recorded after increasing illumination time on an OLIS Co. update of a Cary 15 spectrophotometer. The reference cell contained all buffer components except enzyme, and thus the recorded spectra do not contain the contribution due to deazariboflavin.

The buffer used for the laser flash photolysis experiments was 5 mM Tris-HCl, pH 8.0, containing 0.6 mM EDTA and approximately 100 μM of 5-dRf (I = 5 mM). In some cases 1 mM PDQ^{2+} was added to the solutions (I = 8 mM). Higher ionic strengths were achieved by the addition of sodium chloride to this buffer. Laser flash photolysis generated 5-deazariboflavin radical (5-dRfH$^\bullet$) which rapidly (<1 μs) underwent electron transfer in the presence of PDQ^{2+} to form $PDQ^{\bullet+}$ or transferred an electron directly to GR in the absence of PDQ^{2+}. Laser flash photolysis experiments were performed anaerobically at room temperature. Laser excitation was carried out with a nitrogen laser pumped dye solution (BBQ 2A386 dye from PRA, 396-nm maximum wavelength). A detailed description of the laser flash apparatus and the method of data collection has been previously described (Przysiecki et al., 1985; Bhattacharyya et al., 1983). All kinetic experiments were performed under pseudo-first-order conditions, in which the protein concentration was in large excess over the amount of 5-dRfH$^\bullet$ (and $PDQ^{\bullet+}$) radical produced per flash. Monophasic kinetic traces were analyzed by hand, by fitting to an exponential curve. Biphasic trace analysis was accomplished by computer fitting, using the SIFIT routine from OLIS Co., Jefferson, Ga. In some cases, nonlinear protein concentration dependencies for k_{obs} values were obtained, implying a mechanism that involved intermediate complex formation [cf. Simondsen et al. (1982)]. The second-order rate constants and the limiting first-order rate constants for GR reduction were evaluated from these data by a nonlinear least-squares computer-fitting procedure as described previously (Simondsen et al., 1982). The estimated error in these values is ±15%. Molecular graphics were done by using an Evans and Sutherland PS390 system and the software package INSIGHT (BIOSYM Technologies, Inc.). We are grateful to James T. Hazzard for his assistance with this aspect of the work.

Results and Discussion

Steady-State Phototitrations. As illustrated in Figure 1a, steady-state photoreduction of GR by the $PDQ^{\bullet+}$ radical was accompanied by a decrease in the two maximum peaks of absorbance of the protein at 370 and 463 nm and a broad band of absorbance developed at wavelengths longer than 510 nm (see also Figure 1a inset). The final spectrum obtained after the phototitration has been previously described as being due to the formation of the two-electron-reduced form of the glutathione reductase (EH_2) (Williams, 1976). As the viologen analogue radical is a one-electron donor molecule, the accumulation of the EH_2 species without the observation of intermediate semiquinone formation suggests either the occurrence of a fast disproportionation reaction of two one-electron-reduced semiquinone molecules leading to the formation of the two-electron-reduced enzyme or a rapid reaction of the semiquinone with $PDQ^{\bullet+}$. As will be shown below, transient kinetic measurements support the latter interpretation. In the presence of high concentrations of $NADP^+$ (Figure 1b), the phototitration results in a decrease of the absorbance corresponding to the band around 463 nm and an increase in absorbance at wavelengths longer than 510 nm and shorter than 420 nm, with the appearance of an additional absorbance peak centered around 410 nm (see also Figure 1b inset). This final spectrum has been previously described as a consequence of the slow formation from an EH_2–$NADP^+$ complex of another stable species which was tentatively identified as the anion semiquinone–$NADP^+$ complex (Bulger & Brandt, 1971). In view of the results of Figure 1a, we conclude that $NADP^+$ can interact directly with GR semiquinone to inhibit its further reduction.

As illustrated in Figure 2a, steady-state photoreduction of GR with the 5-dRfH$^\bullet$ radical (i.e., in the absence of PDQ^{2+}) results in the formation of a species whose spectrum has peaks at 360, 400, and 480 nm. These bands correspond closely to those of the well-known anion semiquinone form previously observed in several reduced flavoproteins (Massey & Palmer, 1966). Further illumination of this species resulted in the bleaching of the 5-deazariboflavin spectrum without changes

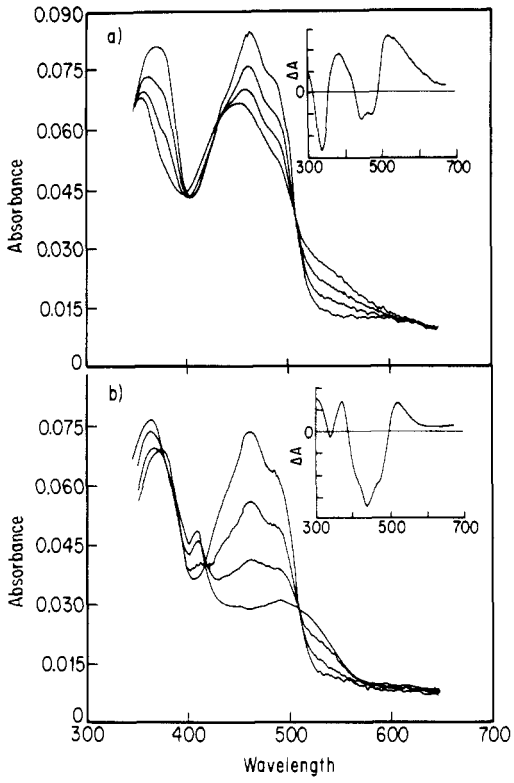

FIGURE 1: Spectral changes observed during steady-state glutathione reductase phototitrations in the presence of PDQ^{2+}. The buffer used was 25 mM Tris-HCl, pH 8.0, containing 1 mM EDTA, 13 μM 5-dRf, and 10 μM PDQ^{2+} in the absence (a) or in the presence (b) of 2 mM $NADP^+$. Protein concentration was 7.5 μM (a) and 7 μM (b). The reference cuvette in the spectrophotometer contained all solution components except glutathione reductase. (Insets) Difference spectra at the end of the phototitrations.

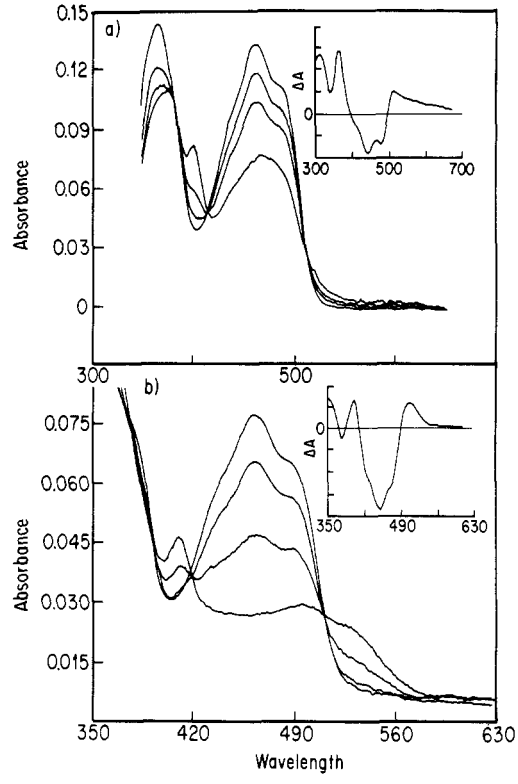

FIGURE 2: Spectral changes associated with steady-state glutathione reductase phototitrations in the presence of 5-deazariboflavin. Experimental conditions were the same as described in Figure 1, except that the experiments were carried out in the absence of PDQ^{2+}. Protein concentration was 12 μM (a) and 7 μM (b). (Insets) Difference spectra at the end of the phototitrations.

in the absorbance due to the protein (data not shown), indicating that the putative anion semiquinone form is stable to further photoreduction. In the presence of an excess of $NADP^+$, steady-state phototritation produced the same spectral changes previously described in the presence of PDQ^{2+} (Figure 2b), indicating that the final species formed in the presence of $NADP^+$ with 5-dRfH$^\bullet$ as reductant is the same as was produced with PDQ^{2+} as the electron donor, i.e., a semiquinone–$NADP^+$ complex. With both reductants, the addition of high concentrations of GSH (the reduced form of the physiological substrate of the enzyme) to the solutions did not alter the course of the phototitrations or the observed final spectra (data not shown).

Laser Flash Induced Reduction of Glutathione Reductase by $PDQ^{\bullet+}$. Glutathione reductase reduction was monitored by loss of absorbance at 463 nm, where the protein has an absorption maximum. The $PDQ^{\bullet+}$ species has broad absorption from 450 to 550 nm, with a peak at about 500 nm [absorptivity is 2.0–3.2 mM^{-1} cm^{-1}; cf. Tsukahara and Wilkins (1985)]. Thus, the kinetic transients contain roughly comparable contributions from both reactants. As illustrated in Figure 3, in the presence of PDQ^{2+} and enzyme at low ionic strength the laser flash resulted in an initial rapid increase in absorbance followed by an exponential decay that on a long time scale went below the preflash base line. Such changes are consistent with the rapid formation of the viologen radical (initial increase) and its subsequent reoxidation (fast absorbance decay) by GR leading to the formation of reduced protein. Two different kinetic processes could be distinguished during this absorbance decay. An initial monoexponential fast phase was observed on short time scales (1–3 ms) (Figure

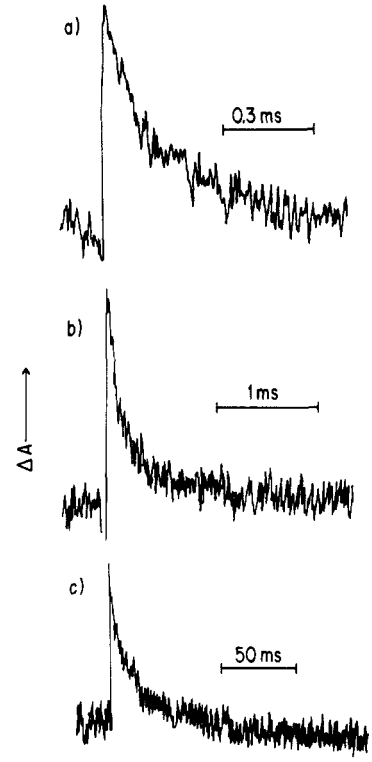

FIGURE 3: Kinetic traces showing glutathione reductase reduction by $PDQ^{\bullet+}$ upon laser flash photolysis, monitored at 463 nm at different time scales. Protein concentration was 19.5 μM. The buffer conditions were 5 mM Tris-HCl, pH 8.0, 0.6 mM EDTA, 1 mM PDQ^{2+}, and 100 μM 5-dRf (I = 8 mM). In the absence of enzyme, only transients corresponding to $PDQ^{\bullet+}$ formation were observed; this species is stable over the time course of these experiments [cf. Figure 1 of Navarro et al. (1989)].

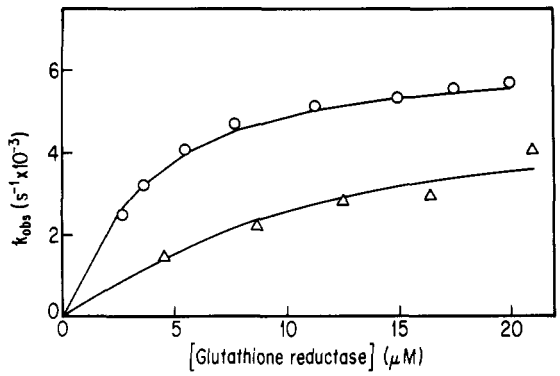

FIGURE 4: Dependence of k_{obs} for glutathione reductase reduction by $PDQ^{\bullet+}$ upon protein concentration at I = 8 mM (○) and I = 408 mM (△). Solid curves correspond to theoretical fits according to the complex formation mechanism given in the text. The buffer conditions were as described under Materials and Methods.

3a,b), followed by a smaller and slower absorbance decay observed at longer times (200 ms) (Figure 3c). The fast phase comprised approximately 80% of the total decay. The observed pseudo-first-order kinetic constants corresponding to the initial fast phase were dependent on the protein concentration (cf. Figure 4), so that this phase was assigned to the formation of the anionic semiquinone species of GR by one-electron transfer from the $PDQ^{\bullet+}$ radical. Observations made at other wavelengths (data not shown) were consistent with this interpretation. The rate of the second slow phase was approximately independent of initial protein concentration (data not shown), although the relatively small signal sizes made this difficult to quantitate. We do not have a definitive explanation at this time for the kinetic process which is responsible for this slow transient phase. However, one possibility is that it results from GR reduction occurring within nonproductive complexes with $PDQ^{\bullet+}$ [cf. Navarro et al. (1989)]. It is important to note that the flash experiments provide no evidence for GR semiquinone disproportionation, which should result in an increase in absorbance at 463 nm.

The protein concentration dependence of the fast kinetic phase of GR reduction by $PDQ^{\bullet+}$ is shown in Figure 4 at two different ionic strengths. It is evident that the observed rate constants for the electron-transfer reaction from the viologen radical to GR decreased at the higher ionic strength. This demonstrates that a negative electrostatic potential exists at or near the site of reaction on the GR molecule. Approximate second-order kinetics were observed at low protein concentration, whereas at higher GR concentration the k_{obs} values became relatively concentration independent. This implies a mechanism consisting of a *minimum* of two steps. The individual rate constants can be obtained by a nonlinear least-squares fit of the data to a simple two-step mechanism, in which the reactants initially form an electrostatically stabilized transient complex followed by intracomplex electron transfer and product formation [cf. Tollin et al. (1986)].

$$A_{ox} + B_{red} \underset{k_{21}}{\overset{k_{12}}{\rightleftharpoons}} \text{complex} \xrightarrow{k_{23}} A_{red} + B_{ox}$$

Such behavior has been previously observed to occur during the electron-transfer reaction between $PDQ^{\bullet+}$ and spinach and *Clostridium* ferredoxins (Navarro et al., 1989). The solid lines in Figure 4 are the theoretical fits according to this mechanism, and as is evident, the agreement with the observed values is satisfactory. In Table I are listed the values of the kinetic constants obtained from the data fitting to this two-step mechanism. The ionic strength effects, although relatively small, are manifested in both the binding step (i.e., k_{12} and K_a) and the product formation step (i.e., k_{23}). This has also been observed previously (Navarro et al., 1989) and has been ascribed to electrostatically promoted orientation of the reactants in a favorable manner for the electron-transfer process.

Table I: Kinetic Constants Corresponding to the Electron-Transfer Reaction from $PDQ^{\bullet+}$ to Glutathione Reductase[a]

ionic strength (mM)	k_{12} (M^{-1} s^{-1})	k_{21} (s^{-1})	K_a (M^{-1})	k_{23} (s^{-1})
8	2.1×10^9	4700	4.5×10^5	6250
408	0.6×10^9	3800	1.6×10^5	5020

[a] The kinetic constants were evaluated from the data presented in Figure 4 by computer fitting according to the mechanism involving intermediate complex formation described in the text [cf. Simondsen et al. (1982)].

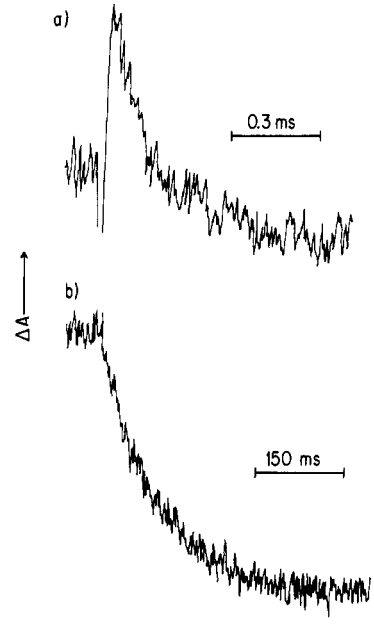

FIGURE 5: Effect of $NADP^+$ on the transient kinetics of glutathione reductase reduction by $PDQ^{\bullet+}$ on short (a) and long (b) time scales. Experimental conditions were the same as described in Figure 3, except that the experiments were carried out in the presence of 2 mM $NADP^+$.

Effects of $NADP^+$ and GSSG on GR Reduction by $PDQ^{\bullet+}$. The presence of excess $NADP^+$ (the oxidized form of the physiological cofactor of GR) did not produce significant changes in the observed kinetics corresponding to the initial fast phase of GR reduction, both at low and high ionic strength, although a small increase in the observed bleaching was obtained (compare Figure 5a with Figure 3a). At longer times the presence of $NADP^+$ resulted in a slow monoexponential absorbance decay which produced an increase in the observed bleaching (Figure 5b; compare with Figure 3c). This bleaching increase was larger at low ionic strength as compared with high ionic strength, although the kinetics themselves were approximately 5 times faster at high ionic strength. The observed rate constant values for this slow phase were also protein concentration dependent (as was the fast phase), and plots of the k_{obs} vs GR concentration yielded second-order rate constants of 0.54×10^6 M^{-1} s^{-1} (low I) and 2.7×10^6 M^{-1} s^{-1} (high I) (not shown). This slow process was also $NADP^+$ concentration dependent, at least at low ionic strength (the data obtained at high ionic strength were not good enough to demonstrate this conclusively). The calculated second-order rate constant obtained from measurements over the concentration range 2–6 mM is 1.3×10^3 M^{-1} s^{-1} (not shown). We

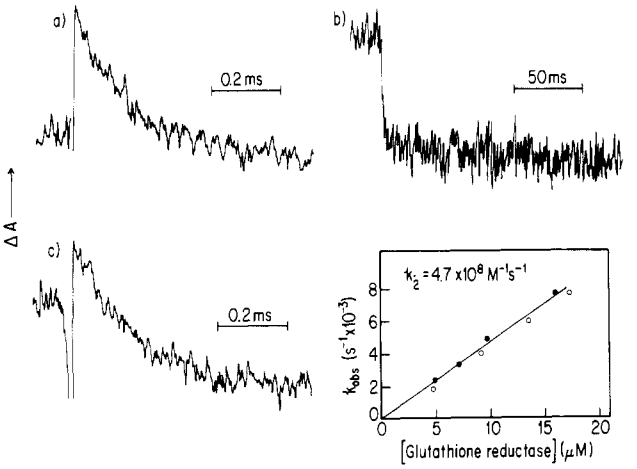

FIGURE 6: Kinetic traces showing glutathione reductase reduction by 5-dRfH$^{\bullet}$ in the absence (a, b) or in the presence (c) of 2 mM $NADP^+$. Experimental conditions were as described in Figure 3, except that the experiments were carried out in the absence of PDQ^{2+}. Protein concentration was 17 μM. (Inset) Dependence of k_{obs} for protein reduction upon glutathione reductase concentration at I = 5 mM (○) and I = 305 mM (●).

assign the slow phase to the formation of an anion semiquinone–$NADP^+$ complex, as described above in the phototitration experiments. The fact that the rate of formation of this species depends on *both* the GR and the $NADP^+$ concentrations suggests that there must be two pathways for its production. A possible mechanism is

$$GR_{ox} + PDQ^{\bullet+} \rightarrow GR_{sq} + PDQ^{2+} \quad (1)$$

$$GR_{sq} + NADP^+ \rightarrow [GR_{sq}\text{–}NADP^+] \quad (2)$$

$$GR_{ox} + NADP^+ \rightleftharpoons [GR_{ox}\text{–}NADP^+] \quad (3)$$

$$[GR_{ox}\text{–}NADP^+] + GR_{sq} \rightarrow [GR_{sq}\text{–}NADP^+] + GR_{ox} \quad (4)$$

In this scheme, reaction 2 accounts for the $NADP^+$ concentration dependence ($k = 1.3 \times 10^3\ M^{-1}\ s^{-1}$), and reactions 3 and 4 account for the GR concentration dependence (the calculated second-order rate constant for this process would then be equal to the product of the equilibrium constant for reaction 3 and the pseudo-first-order rate constant for reaction 4). If these two pathways have comparable rates under the conditions of our experiments, biphasic kinetics would not be expected.

In contrast to the results with $NADP^+$, the addition of high concentrations of GSSG (>2 mM) had no significant effect on the kinetics of GR reduction on either slow or fast time scales, both at high and low ionic strength (data not shown).

Kinetics of Reduction of GR by 5-dRfH$^{\bullet}$. GR reduction by 5-dRfH$^{\bullet}$ (in the absence of PDQ^{2+}) was again monitored by the absorbance decrease at 463 nm, where the oxidized protein has an absorption maximum. In the presence of GR at low ionic strength (Figure 6a), 5-dRf-sensitized laser photolysis showed an initial increase in absorbance followed by an exponential fast decay (1–3 ms) that eventually went below the preflash base line. In contrast to the results with PDQ^{2+}, no further absorbance changes could be detected at longer times (Figure 6b), again indicating the absence of semiquinone disproportionation on this time scale. These results are consistent with the rapid formation of 5-dRfH$^{\bullet}$ followed by its subsequent reoxidation by GR, leading to the formation of the semiquinone species of the enzyme, as previously described in the phototitration experiments. From the slope of a plot of k_{obs} vs GR concentration, a second-order rate constant for the enzyme reduction of $4.7 \times 10^8\ M^{-1}\ s^{-1}$ was obtained. This value was independent of ionic strength changes (see Figure 6, inset), in agreement with the fact that the 5-dRfH$^{\bullet}$ radical is an uncharged electron donor.

The steady-state phototitration results indicate that the semiquinone reduction reaction which leads to the formation of EH_2 is inhibited when 5-dRfH$^{\bullet}$ is used as the electron source in the absence of PDQ^{2+}. Why this is so is not entirely clear at this time. However, the fact that complexation with $NADP^+$ also prevents this reaction (see above) suggests that perhaps complexation effects are occurring here as well, e.g., complex formation between the GR semiquinone and 5-dRf. This would have to be prevented by an interaction between the GR semiquinone and PDQ^{2+} which does not inhibit EH_2 formation. This requires further investigation. It is of interest to note that a phenazine derivative (safranin; 3,7-diamino-2,8-dimethyl-5-phenylphenazinium chloride) and menadione have recently been found to bind in a large cavity at the dimer interface of GR (Karplus et al., 1989). Both of these compounds have polycyclic ring structures, as do 5-dRf and PDQ^{2+}.

$NADP^+$ had only a slight effect on the GR reduction kinetics with 5-dRfH$^{\bullet}$. The addition of high concentrations of $NADP^+$ caused a small increase in the observed bleaching (Figure 6c), with no effect on the kinetic constant values themselves. No differences were detected on a long time scale in the presence or in the absence of $NADP^+$ (data not shown). This implies that the reaction of the GR semiquinone with $NADP^+$ is much faster when 5-dRfH$^{\bullet}$ is the electron donor than is the case when $PDQ^{\bullet+}$ is the reductant. Thus, although according to the phototitration experiments the same spectral species is formed in the presence of $NADP^+$ with either 5-dRfH$^{\bullet}$ or $PDQ^{\bullet+}$ as electron donors, in fact in each case the reactivity of the GR semiquinone toward the oxidized pyridine nucleotide is apparently different. This again suggests complexation effects on the properties of this species.

A different approach was used to test if the semiquinone–enzyme species formed upon GR reduction with 5-deazariboflavin is indeed a dead-end complex, as the laser flash and the steady-state experiments suggest. Thus, a 5-deazariboflavin GR sample was phototitrated until the spectrum corresponding to the semiquinone complex appeared. After this had occurred, the sample showed no reactivity upon laser flash photolysis toward the 5-dRfH$^{\bullet}$ radical (data not shown).

As was previously described in the case of GR reduction with $PDQ^{\bullet+}$, the addition of high concentrations of GSSG had no effect on the kinetics of GR reduction by 5-dRfH$^{\bullet}$, both at low and high ionic strength (data not shown).

Conclusions

The results described above provide clear evidence for one-electron reduction of glutathione reductase to form a semiquinone species which can be stabilized against further reduction by complexation with $NADP^+$, and possibly also with 5-deazariboflavin. The results also demonstrate that introduction of an electron into the molecule by low-potential reductants occurs at close to diffusion-controlled rates (second-order rate constants in the range of 10^8–$10^9\ M^{-1}\ s^{-1}$), despite the relatively buried nature of the FAD cofactor (Karplus & Schulz, 1987a), that the site of reduction carries a negative electrostatic potential, and that entry of the electron is not affected by complexation of the protein with $NADP^+$ or GSSG. It is of interest that previous steady-state kinetic measurements (Llobell et al., 1986) have indicated that methylviologen radical does not interact with the pyridine nucleotide binding site of GR. The X-ray crystal structure of human erythrocyte GR (Karplus & Schulz, 1987a) indicates

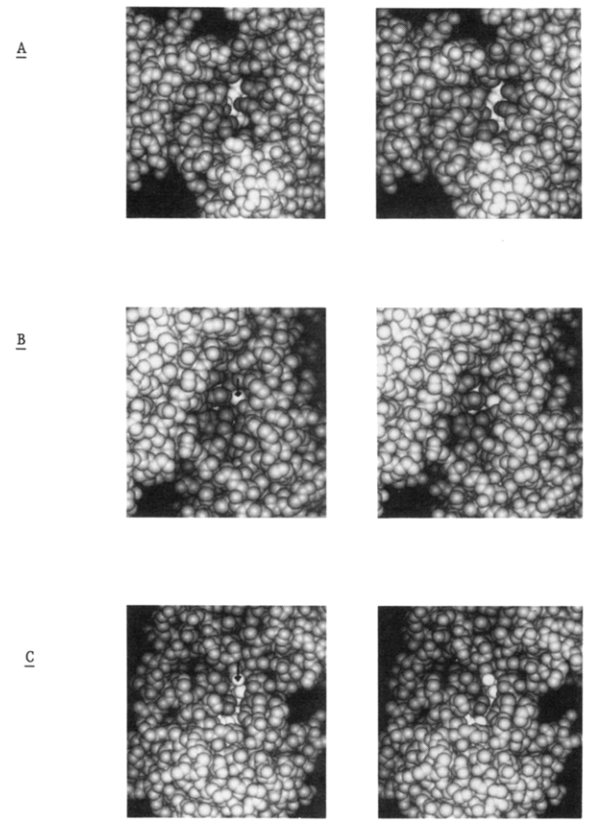

FIGURE 7: Stereoviews of glutathione reductase showing FAD exposure. (A) NADPH binding site. (B) Glutathione binding site. (C) Site of dimethylbenzene ring exposure. The lightest colored atoms in the central background of each view are the exposed FAD atoms. View B also shows one of the sulfur atoms of the redox-active disulfide (lightest colored atom at right center; see arrow), and view C shows the carboxyl oxygens of Asp-178 (lightest colored atoms at top center; see arrow).

that the FAD cofactor is exposed to the solvent at three locations in the molecule (Figure 7): the NADPH binding site (which has a moderately large degree of exposure), the GSSG binding site (at which the exposure is quite small), and a site that is located approximately 90° from these two substrate sites, on the side of the molecule opposite to the second subunit (at which the dimethylbenzene end of the flavin ring has an intermediate degree of exposure). We suggest that electron entry into the molecule from either $PDQ^{\bullet+}$ or $5\text{-dRfH}^{\bullet}$ occurs at this third site, based on the lack of effect of substrate binding, and also on the negatively charged side chains which are located in this region of the molecule (Asp-178 and possibly also Asp-297). Apparently, sufficient flavin exposure exists at this site to allow rapid reduction, at least by relatively small molecules. Whether or not this site of reduction has any biological significance remains to be determined. This identification assumes, of course, structural homology between the yeast and human enzymes. Unfortunately, a complete amino acid sequence is not available for the yeast GR.

In none of the flash photolysis experiments reported above was there any indication in the kinetic traces of intramolecular electron transfer between the FAD and the protein disulfide. Thus, if this does indeed occur in the semiquinone form of the enzyme, then the rate constant must be appreciably larger than 8000 s^{-1} (Figure 6). This would not be surprising in view of the close proximity of these two centers in the GR molecule (Karplus & Schulz, 1987a).

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