Flavin Fluorescence Dynamics and Photoinduced Electron Transfer in *Escherichia coli* Glutathione Reductase

Petra A. W. van den Berg,* Arie van Hoek,* Christopher D. Walentas,# Richard N. Perham,# and Antonie J. W. G. Visser*

*MicroSpectroscopy Centre, Department of Biomolecular Sciences, Wageningen Agricultural University, Wageningen, The Netherlands, and #Cambridge Centre for Molecular Recognition, Department of Biochemistry, University of Cambridge, Cambridge, England

ABSTRACT Time-resolved polarized flavin fluorescence was used to study the active site dynamics of *Escherichia coli* glutathione reductase (GR). Special consideration was given to the role of Tyr^{177}, which blocks the access to the NADPH binding-site in the crystal structure of the enzyme. By comparing wild-type GR with the mutant enzymes Y177F and Y177G, a fluorescence lifetime of 7 ps that accounts for ~90% of the fluorescence decay could be attributed to quenching by Y177. Based on the temperature invariance for this lifetime, and the very high quenching rate, electron transfer from Y177 to the light-excited isoalloxazine part of flavin adenine dinucleotide (FAD) is proposed as the mechanism of flavin fluorescence quenching. Contrary to the mutant enzymes, wild-type GR shows a rapid fluorescence depolarization. This depolarization process is likely to originate from a transient charge transfer interaction between Y177 and the light-excited FAD, and not from internal mobility of the flavin, as has previously been proposed. Based on the fluorescence lifetime distributions, the mutants Y177F and Y177G have a more flexible protein structure than wild-type GR: in the range of 223 K to 277 K in 80% glycerol, both tyrosine mutants mimic the closely related enzyme dihydrolipoyl dehydrogenase. The fluorescence intensity decays of the GR enzymes can only be explained by the existence of multiple quenching sites in the protein. Although structural fluctuations are likely to contribute to the nonexponential decay and the probability of quenching by a specific site, the concept of conformational substates need not be invoked to explain the heterogeneous fluorescence dynamics.

INTRODUCTION

Increasingly, protein flexibility and conformational dynamics are considered to play a role in the catalytic mechanism of enzymes (Careri et al., 1979; Welch et al., 1982; Karplus and McCammon, 1983; Frauenfelder et al., 1988). Yet visualizing protein motions by experimental methods is still a little explored field. In enzymes containing an intrinsic fluorescent group like Trp, protein dynamics of the fluorophore environment can be monitored by time-resolved fluorescence and fluorescence anisotropy detection (Millar, 1996). Flavoproteins, which have either flavin adenine dinucleotide (FAD) or flavin mononucleotide (FMN) as a redox-active prosthetic group, offer the unique possibility of probing the dynamical behavior of the active site via this specific cofactor. The fluorescence characteristics of the isoalloxazine ring of the cofactor vary highly among different flavoproteins, thus reflecting structural and dynamical differences near the active site. In this study polarized time-resolved flavin fluorescence is used to monitor the active-site dynamics of *Escherichia coli* glutathione reductase.

Glutathione reductase (GR; EC 1.6.4.2) belongs to the pyridine nucleotide disulfide-oxidoreductase family, which includes, among others, dihydrolipoyl dehydrogenase and thioredoxin reductase. The enzyme catalyzes the NADPH-dependent reduction of oxidized glutathione (Williams, 1976). The primary function of glutathione reductase is to maintain a high GSH/GSSG ratio in cells, which is crucial for a variety of cellular functions, including the biosynthesis of DNA (Holmgren, 1985). Glutathione reductase is a homodimeric enzyme with a molecular mass of ~50 kDa per subunit. The enzyme contains one molecule of FAD per subunit. This flavin cofactor is noncovalently bound, and is trapped in a tight binding-site between the two subunits. The kinetic mechanism of glutathione reductase from various sources has been studied extensively. The native enzyme acts according to a ping-pong mechanism, but branched ping-pong and ordered sequential mechanisms have been suggested for specific mutants and for high concentrations of GSSG (Williams, 1992). Extensive site-directed mutagenesis experiments with *E. coli* GR have resulted in mutants that yield information on the mechanism and substrate and coenzyme specificity (Berry et al., 1989; Deonarain et al., 1989, 1990; Scrutton et al., 1990; Bashir et al., 1995).

Detailed structural information on glutathione reductase from both human erythrocytes and *E. coli* is available from high-resolution crystal structures (Thieme et al., 1981; Karplus and Schulz, 1987, 1989; Mittl and Schulz, 1994; Ermler and Schulz, 1991). Although the enzymes have only 52% sequence identity, and the 16 N-terminal residues of erythrocyte glutathione reductase are missing in the *E. coli* enzyme (Greer and Perham, 1986), the tertiary structures reveal little significant difference. From a mechanistic point of view, one of the most compelling structural characteris-

Received for publication 23 September 1997 and in final form 19 January 1998.

Address reprint requests to Dr. A. J. W. G. Visser, MicroSpectroscopy Centre, Department of Biomolecular Sciences, Laboratory of Biochemistry, WAU, Dreijenlaan 3, 6703 HA Wageningen, The Netherlands. Tel.: 31-317-482862; Fax: 31-317-484801; E-mail: ton.visser@laser.bc.wau.nl.

0006-3495/98/04/2046/13 $2.00

tics is the position of the tyrosine adjacent to the flavin (Y177 in *E. coli* GR and Y197 in erythrocyte GR). From both crystal structures it is clear that this tyrosine residue blocks the active site, thereby preventing the binding of NADPH ("in" position). The catalytic mechanism, which was based on three-dimensional structures of free and substrate-bound forms of the human enzyme, therefore includes a movement of this tyrosine away from the flavin ("out" position) (Pai and Schulz, 1983).

To explore the role of this flavin-shielding tyrosine, Berry et al. (1989) used site-directed mutagenesis to change Y177 of the *E. coli* enzyme. Kinetic analysis of these mutants showed that a Y177F mutation hardly affected the enzymatic activity. The activity of the Y177G mutant, however, was significantly diminished. For both mutants an increase in fluorescence intensity of ~25-fold with respect to that of the wild-type enzyme was observed. A previous time-resolved fluorescence study of wild-type glutathione reductase from human erythrocytes showed considerable quenching of the flavin fluorescence, presumably caused by interaction with Y197 (Bastiaens et al., 1992b).

In that same study, the heterogeneous fluorescence decay of the flavin in erythrocyte glutathione reductase was explained by the existence of distinct conformational substates of the enzyme (Bastiaens et al., 1992b). The model of conformational substates is based on the idea that a protein in a certain state has a wide variety of nearly isoenergetic conformational substates, which perform the same function but at different rates (Frauenfelder and Gratton, 1986; Frauenfelder et al., 1988, 1991). Between the different substates energy barriers prevail, the height of which is assumed to be correlated with the extent of protein motion that is involved with interconversion. If different conformational substates result in inhomogeneity of the chromophore environment, excitation will lead to different excited-state processes and consequently to nonexponential fluorescence decay (Beechem and Brand, 1985). Such a heterogeneous fluorescence decay can be described by a multiexponential model or a quasicontinuous distribution of lifetimes (Alcala et al., 1987; Siemiarczuk et al., 1990; Ferreira et al., 1994; Bismuto et al., 1996). When conformational transitions take place during the lifetime of the excited state, the fluorophore will sample a variety of environments, which also results in a distribution of fluorescence lifetimes (Alcala et al., 1987).

In explaining fluorescence decay in terms of conformational substates, however, it is assumed that in each substate the interactions between the chromophore and its direct environment will result in a single fluorescence lifetime. This model has been challenged by Bajzer and Prendergast (1993), who have proposed that the heterogeneity in fluorescence decay can arise from a multiplicity of competing interactions that involve transfer of energy in a broad sense from the light-excited chromophore to different sites in the molecule (multiple quenching sites). They showed that the nonexponential fluorescence decay of several tryptophan-containing proteins can be explained by energy transfer to different acceptor sites in the protein, which all contribute with a certain probability to deexcitation of the donor. In contrast to the conformationally determined models of quenching, which all assume energy transfer via collisional quenching, the model of multiple quenching sites is not dependent on collisions, but includes other deexcitation processes such as fluorescence resonance energy transfer and electron transfer. A multiexponential model for the fluorescence decay can then be justified without invoking multiple protein conformations.

This paper focuses on the active site dynamics of *E. coli* glutathione reductase, and the role of Y177, which shields the flavin part of the FAD. For this, comparison of wild-type *E. coli* GR with site-directed mutants of Y177 (Y177F and Y177G) is indispensable. Variations in temperature and concentration of the cosolvent glycerol are used as complementary approaches to influence protein dynamics. Absorption spectra serve to visualize possible changes in the direct microenvironment of the flavin. The fluorescence lifetime distributions are discussed in terms of the proposed models that account for multiexponential fluorescence decay kinetics.

MATERIALS AND METHODS

Purification and preparation of wild-type and mutant glutathione reductase

Wild-type and mutant glutathione reductases were purified from the *gor*-deleted *E. coli* strains NS3 and SG5, respectively, which were transformed with the appropriate expression plasmid (Scrutton et al., 1987). The purification was based on the method described by Berry et al. (1989), with a modification similar to that described by Bashir et al. (1995). Instead of the DE-52 column, Highload Q (Pharmacia) was used for ion exchange, but the elution conditions were unaltered. Except for the preparation of the cell extract, no additional FAD or EDTA was used. GR activity was measured as described by Scrutton et al. (1987). During the purification, fractions were also tested for dihydrolipoyl dehydrogenase activity (Benen et al., 1991), to ensure that no trace of this more fluorescent, iso-molecular-weight protein contaminated the samples. The enzyme preparations were pure as judged by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and fluorescence detection of the proteins on a nondenaturing gel. Pure enzyme was stored in 80% ammonium sulfate at 277 K. Before use, possible traces of free FAD were removed by chromatography on a Biogel PGD-6 column (Biorad) equilibrated with phosphate buffer. In all spectroscopic measurements the enzyme concentration was 10 μM with respect to FAD. The final potassium phosphate concentration was kept at 50 mM, and the pH at 7.6 (293 K). Buffers were made from nanopure-grade water (Millipore) and were filtered through a 0.22-μm filter (Millipore). All chemicals used were of the highest purity available. Samples containing glycerol were prepared by gently mixing the eluted protein with fluorescent-grade glycerol (Merck). Before and after a series of fluorescence experiments, flavin absorption spectra (Aminco DW2000 spectrophotometer; SLM Instruments, Urbana, IL) were measured at 298 K to check the quality of the samples.

Time-resolved fluorescence and fluorescence anisotropy measurements

Time-resolved polarized fluorescence experiments were carried out using the time-correlated single photon counting technique (TCSPC) (O'Connor and Phillips, 1984). The TCSPC set-up used was basically as described in detail elsewhere (Bastiaens et al., 1992b), with a few modifications. A mode-locked CW Nd:YLF laser (model Antares 76-YLF; Coherent, Santa

Clara, CA) was used for the synchronous pumping of a cavity-dumped (Coherent Radiation model CR590) dye laser with Stilbene 420 (Exciton, Inc., Dayton, OH) as a dye (van Hoek and Visser, 1992). The samples were excited with vertically polarized light of 450 nm, with an excitation frequency of 594 kHz and a duration of 4 ps full width at half-maximum. Parallel and perpendicular polarized fluorescence was detected with a 557.9-nm interference filter (half-bandwidth of 11.8 nm; Schott, Mainz, Germany) in combination with a KV 520 cutoff filter. Time-resolved fluorescence experiments with sucrose were carried out at a later date, using Coumarin 460 (Exciton) as a dye and an excitation wavelength of 460 nm. To avoid possible distortions caused by Raman scattering of water, fluorescence here was detected with a 526.0-nm interference filter (half-bandwidth of 12.6 nm; Schott) in combination with a KV 520 cutoff filter. A turbid glycogen solution (OD 0.1 at 450 nm) was used to verify that the data were free of scattered excitation light. Fluorescence was sampled in cycles of 10 s in each polarization direction. By adjusting the excitation energy, the detection frequency of the parallel polarized component was set to 30 kHz to prevent pile-up distortion. Detection electronics were standard time correlated single photon counting modules. The presented data were collected in a multichannel analyzer (MCA board from Nuclear Data model AccuspecB, Schaumburg, IL) with a time window of 1024 channels at 15.1 or 16.7 ps/channel respectively. As a control, data of wild-type GR were also collected at 5.0 ps/channel. Instrumental sources for distortion of data were minimized to below the noise level of normal photon statistics (van Hoek and Visser, 1985).

The dynamic instrumental response function of the set-up is ~50 ps FWHM. The instrumental response was obtained at the emission wavelength by measuring a reference compound with a known single fluorescence lifetime (Vos et al., 1987). As a reference compound in the range between 277 K and 313 K, erythrosine B in water ($\tau = 80$ ps at 293 K) was used. In the range between 293 K and 223 K, erythrosine B was dissolved in methanol, yielding a fluorescence lifetime of ~500 ps (Bastiaens et al., 1992b). To correct for the small temperature dependence of the reference lifetime, the exact value at a certain temperature was determined by iterative reconvolution with the reference compound at a temperature at which the lifetime was known. In each experiment, the parallel and perpendicular fluorescence decay components of the reference compound (3 cycles), the sample (10 or 15 cycles), the background (one-fifth of the sample acquisition time), and again the reference compound were determined. The temperature of the samples was controlled with a liquid nitrogen flow set-up with a temperature controller (model ITC4; Oxford Instruments, Oxford, England). At temperatures below 273 K, the sample housing was flushed with argon to prevent dew and ice formation (Bastiaens et al., 1992a).

Data analysis

The fluorescence intensity decay $I(t)$ and anisotropy decay $r(t)$ were analyzed by the commercially available maximum entropy method (MEM) (Maximum Entropy Data Consultants, Cambridge, England). With this method, the fluorescence intensity and anisotropy decays can be described in terms of a continuous distribution of decay times, for which no a priori knowledge of the system is required. A detailed description of the principles of MEM and analysis of the polarized fluorescence data can be found elsewhere (Livesey and Brochon, 1987; Bastiaens et al., 1992b; Brochon, 1994) and will be shortly outlined below. In the experiments the parallel $I_{\parallel}(t)$ and perpendicular $I_{\perp}(t)$ fluorescence intensity components were acquired separately after excitation with a vertically polarized light pulse. The lifetime spectrum $\alpha(\tau)$ was obtained from the total fluorescence $I(t)$ via the inverse Laplace transform

$$I(t) = I_{\parallel}(t) + 2gI_{\perp}(t) = E(t)^* \int_0^{\infty} \alpha(\tau) e^{-t/\tau}\, d\tau \qquad (1)$$

where $E(t)$ is the instrumental response function, and the factor g describes the sensitivity of the detection system for the perpendicular component relative to the parallel one. In our TCSPC set-up, this g-factor equals 1 (van Hoek et al., 1987). In the analysis, the starting model for the distribution of lifetimes was chosen to be flat in $\log(\tau)$ space, as no a priori knowledge was introduced. The lifetime spectrum $\alpha(\tau)$ that was recovered consisted of 150 decay times equally spaced in $\log(\tau)$ space between 1 ps and 10 ns. As no density filters were needed, the optical paths of reference and samples were identical. No indications of the existence of significant amounts of scattered light were found. The average fluorescence lifetime $\langle\tau\rangle$ was calculated from the lifetime spectrum $\alpha(\tau)$ according to

$$\langle\tau\rangle = \frac{\sum_{i=1}^{N} \alpha_i \tau_i}{\sum_{i=1}^{N} \alpha_i} \qquad (2)$$

where N is the number of τ_i values of the $\alpha(\tau)$ spectrum. The barycenter of a peak is determined in a similar fashion, with the summation carried out over a limited range of τ_i values encompassing a local peak (Mérola et al., 1989).

In a similar way, a rotational correlation time spectrum $\beta(\phi)$ was obtained from the anisotropy decay $r(t)$, which is defined as

$$r(t) = \frac{I_{\parallel}(t) - I_{\perp}(t)}{I_{\parallel}(t) + 2I_{\perp}(t)} \qquad (3)$$

Under the assumption that the fluorescence lifetime and the rotational correlation time (ϕ) are uncorrelated, the time dependence of the anisotropy after δ-pulse excitation can be described as

$$r(t) = \int_0^{\infty} \beta(\phi) e^{-t/\phi}\, d\phi \qquad (4)$$

where the integrated amplitude $\beta(\phi)$ corresponds to the initial anisotropy r_0. In the MEM analysis of the anisotropy, 100 decay times equally spaced in $\log(\phi)$ space between 0.01 and 100 ns were introduced. The starting model for the rotational correlation spectra was again chosen to be flat, as no a priori knowledge was introduced.

RESULTS

Flavin fluorescence intensity decay

Both the wild-type glutathione reductase (GR) and the mutants GR Y177F and Y177G showed a very rapid fluorescence decay with respect to a nonquenched flavin compound such as FMN in aqueous solution (Fig. 1). The fluorescence of wild-type GR was obviously the most quenched, which is reflected in an average lifetime of only 27 ps for wild-type enzyme, which is approximately five to six times shorter than that of the tyrosine mutants (Table 1). Analysis of the fluorescence decays of the enzymes resulted in heterogeneous lifetime distributions containing five distinct components varying in the range between 7 ps and 3 ns (Fig. 2). Further analysis of the decay by least-squares fitting of the data using global analysis with a sum of exponentials revealed that all five components were needed for a satisfactory approximation of the fluorescence decay. The positions of the four longer lifetimes were almost invariant for the different enzymes, but the relative contributions differed highly (Table 2). In wild-type GR, the ultrafast component of 7 ps was predominant (~90%). The uncertainty in the position of this lifetime was only 1 ps. In the case of the mutants, a longer component of ~90 ps was responsible for ~40–50% of the fluorescence intensity de-

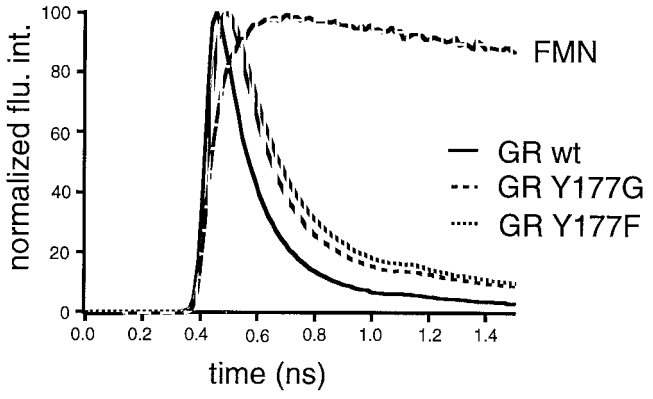

FIGURE 1 Experimental total fluorescence decay of wild-type GR and mutants Y177F and Y177G in 50 mM potassium phosphate buffer (pH 7.6) and FMN in 50 mM potassium phosphate buffer (pH 7.0) at 293 K. Only the initial part of the 16-ns experimental time window is shown.

cay. In these lifetime distributions the 7-ps component disappeared. Tyr^{177} could thus be identified as the residue responsible for the extremely efficient fluorescence quenching of the flavin in wild-type *E. coli* glutathione reductase.

Varying the temperature between 223 K (in 80% v/v glycerol) and 313 K did not affect the position of the 7-ps component in wild-type GR. Collisional quenching can therefore be excluded. Based on this temperature invariance and the ultrafast lifetime, we propose electron transfer as the mechanism of fluorescence quenching. In the excited state the flavin may abstract an electron from Y177; return to the ground state may then occur via a radiationless process. The edge-to-edge distance between FAD and the electron-rich Y177, as found in the crystal structure of *E. coli* GR, is 3.1 Å, thus allowing fast electron transfer (Moser et al., 1992).

Supporting evidence for a strong interaction between FAD and Y177 was found in the absorption spectra of the enzymes (Fig. 3 *A*). In the absorption spectrum of wild-type GR, a pronounced shoulder was present at the red-edge side of the first absorption band of FAD, near 490 nm. This shoulder reflects the 0,0 absorption transition ($S_{0,0} \rightarrow S_{1,0}$). Although the mutation from tyrosine to phenylalanine does not change the aromatic character of residue 177, and even increases its hydrophobicity, this shoulder was substantially decreased in the spectrum of Y177F. Mutation into a glycine residue also gave rise to a diminished shoulder at 490 nm and, in addition, to a blueshift of the absorption maximum from 462 nm to 458 nm.

TABLE 1 Average fluorescence lifetimes $\langle\tau\rangle$ of wild-type GR and mutants Y177F and Y177G in 50 mM potassium phosphate buffer (pH 7.6) at 293 K with 0% or 80% (v/v) glycerol

GR	$\langle\tau\rangle$ 0% (ns)	$\langle\tau\rangle$ 80% (ns)
Wild type	0.027 ± 0.003	0.38 ± 0.03
Y177F	0.18 ± 0.004	1.8 ± 0.08
Y177G	0.12 ± 0.017	1.3 ± 0.3

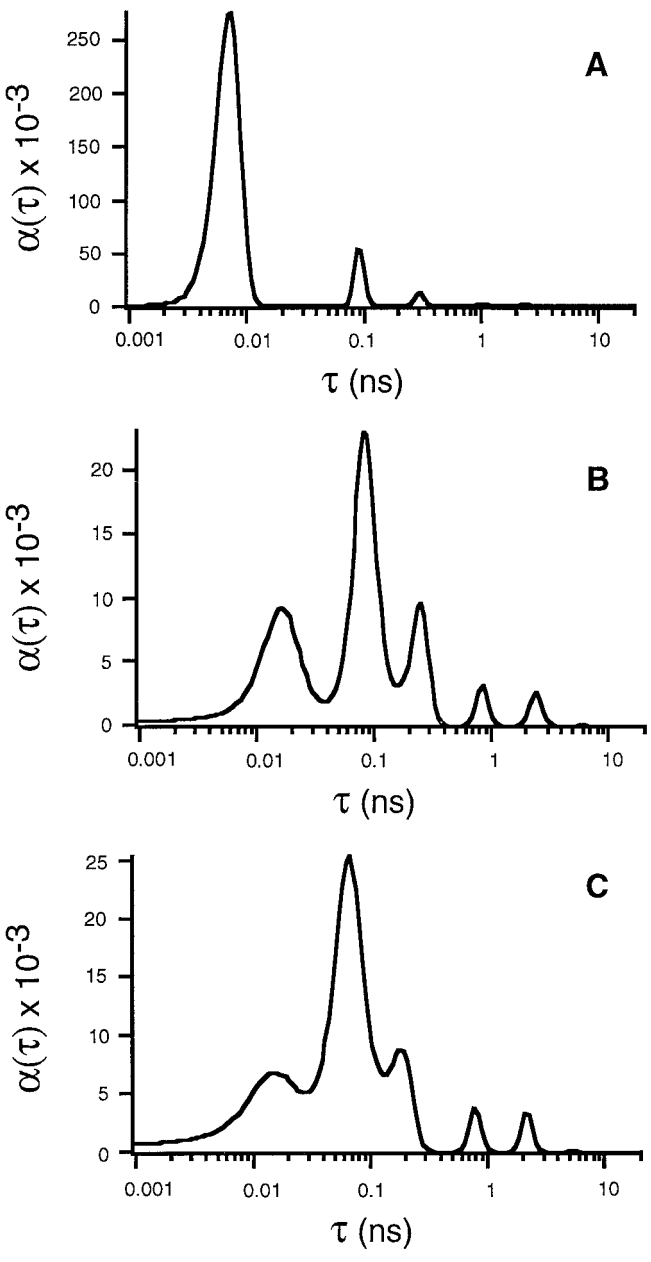

FIGURE 2 Fluorescence lifetime distribution of wild-type GR (*A*) and mutants Y177F (*B*) and Y177G (*C*) in 50 mM potassium phosphate buffer (pH 7.6) at 293 K.

More evidence for this interaction was found upon addition of the cosolvent glycerol. Contrary to the absorption spectrum of the tyrosine mutants, the shape of the absorption spectrum of wild-type GR showed a clear dependence on the glycerol concentration. An increase in the glycerol concentration up to 40% did not bring about any difference. However, at higher concentrations (≥60%), the shape of the absorption spectrum was altered (Fig. 3 *B*): in 80% glycerol the spectrum of wild-type GR showed a diminished shoulder at 490 nm, now more resembling that of GR Y177F in aqueous solution. These results are in accord with time-resolved fluorescence measurements of wild-type GR in

TABLE 2 Barycenters and fractional contributions of the fluorescence lifetime components of wild-type GR and mutants Y177F and Y177G in 50 mM potassium phosphate buffer (pH 7.6) at 293 K

GR wild-type		GR Y177F		GRY177G	
τ_i (ns)	α_i	τ_i (ns)	α_i	τ_i (ns)	α_i
0.007 ± 0.001	0.90 ± 0.01	0.020 ± 0.005	0.34 ± 0.01	0.023 ± 0.01	0.44 ± 0.1
0.090 ± 0.008	0.076 ± 0.005	0.087 ± 0.01	0.41 ± 0.05	0.10 ± 0.004	0.42 ± 0.09
0.29 ± 0.04	0.020 ± 0.005	0.24 ± 0.03	0.19 ± 0.03	0.26 ± 0.006	0.10 ± 0.02
1.0 ± 0.2	0.003 ± 0.001	0.8 ± 0.04	0.03 ± 0.004	0.79 ± 0.007	0.02 ± 0.005
2.6 ± 0.3	0.002 ± 0.0005	2.5 ± 0.1	0.02 ± 0.001	2.2 ± 0.05	0.02 ± 0.003

80% glycerol. The lifetime pattern of the wild-type enzyme again contained the ultrashort component, but its contribution was substantially reduced (Fig. 4).

The average lifetime increased by a factor of more than 10 (Table 1). Using high concentrations of sucrose (50–60% w/w) to increase the solvent viscosity to the same order of magnitude as 80% glycerol did not affect the absorption spectrum or the fluorescence lifetime distribution of wild-type glutathione reductase. A minor shift in the lifetime contributions—favoring the 90-ps component over the 7-ps component—was observed, which may reflect the influence of the increase in internal friction of the enzyme ($\langle\tau\rangle = 0.06$ ns). The interaction between FAD and Y177 thus seemed to be specifically perturbed in high concentrations of glycerol, leading to the presence of unfavorable conformations for efficient electron transfer to the light-excited flavin.

Protein dynamics in *E. coli* glutathione reductase

The fluorescence lifetime spectrum of FAD in wild-type GR was almost independent of temperature in the range between 277 K and 313 K (Fig. 5 *A*). Using 80% glycerol, the temperature of the samples could be varied over a wider temperature range (223–293 K). In this range more significant differences in the contributions of the five lifetimes were found (Fig. 5 *B*). The largest effect on the fluorescence decay patterns was caused by the addition of high concentrations of glycerol itself (Fig. 4). Up to a concentration of 40% glycerol the lifetime patterns remained identical. In 80% glycerol, however, the amplitude of the ultrashort

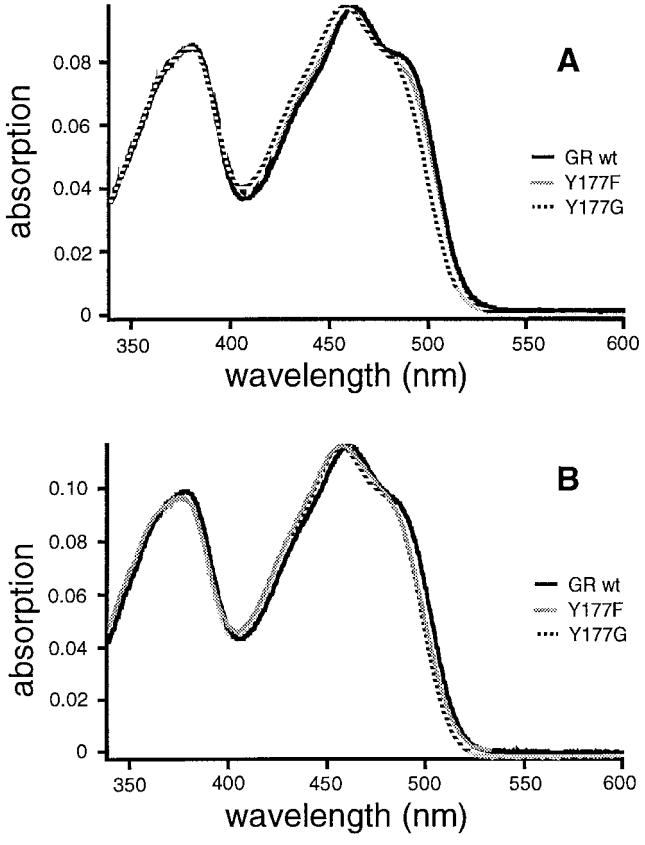

FIGURE 3 Absorption spectra of wild-type GR and mutants Y177F and Y177G in 50 mM potassium phosphate buffer (pH 7.6) with 0% (*A*) and 80% (*B*) glycerol (v/v) at 293 K.

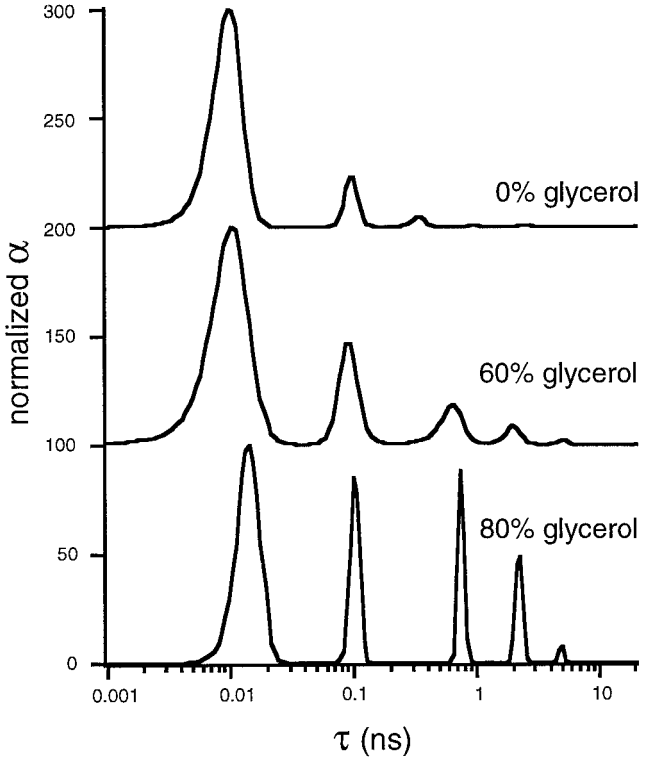

FIGURE 4 Normalized fluorescence lifetime distribution of wild-type GR in 50 mM potassium phosphate (pH 7.6) at 293 K as a function of glycerol concentration. For clarity, a vertical offset has been applied.

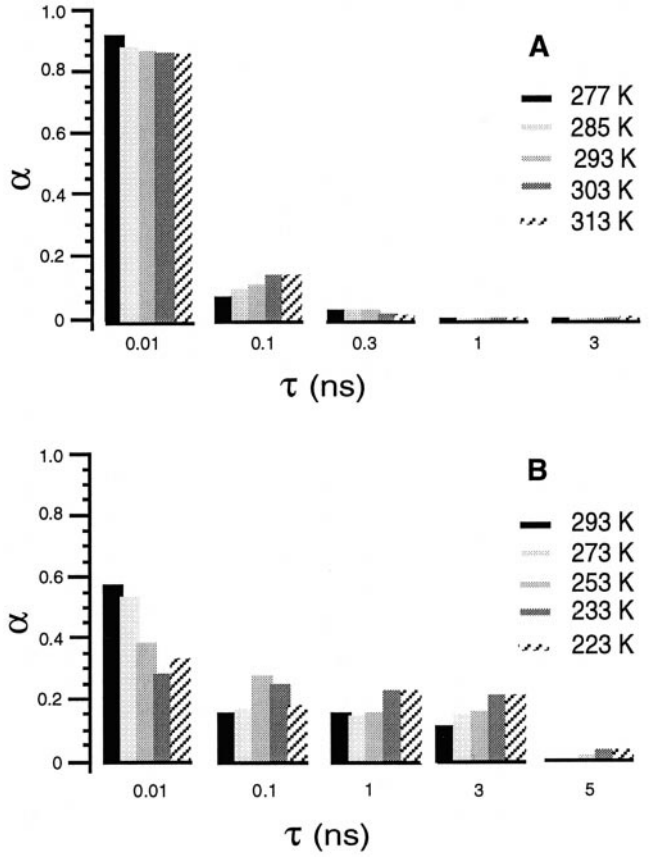

FIGURE 5 Temperature dependence of the fractional contributions of the lifetime classes of wild-type GR in 50 mM potassium phosphate buffer (pH 7.6) in the range between 277 and 313 K (*A*), and with 80% glycerol (v/v) in the range between 223 and 293 K (*B*). For reasons of clarity, the data presented are rounded off to the most significant digit.

component drastically decreased (to ~55%), in favor of the contribution of the two longest lifetimes (Fig. 4). The lifetime component at 0.3 ns was no longer resolved. Combined with the absorption spectra described, the decrease in the 7-ps component was interpreted as a perturbation of the interaction between the light-excited flavin and Y177.

From the temperature independence it can be concluded that wild-type *E. coli* glutathione reductase has a very rigid structure in the immediate vicinity of the flavin. In contrast to this is the dynamical behavior of the tyrosine mutants GR Y177F and Y177G. The lifetime spectra of these enzymes are much more dependent on both temperature and glycerol concentration. In aqueous solution, a rise in temperature favored the fractional contribution of the shorter lifetimes, and the barycenters shifted to somewhat shorter values. As the lifetime positions do not converge upon the rise in temperature, which would be typical for interconversion between different substates, we find the results more indicative of an increased flexibility of the protein matrix of these mutants. A drastic change in the lifetime profiles was found in high concentrations of glycerol. In 80% glycerol, only three discrete lifetimes could be distinguished for both GR Y177F and Y177G. Increasing the temperature from 277 K to 313 K again favored the contribution of the shortest lifetime, and clearly shifted the barycenters (Fig. 6). In these lifetime spectra, a small contribution (less than 5%) of a lifetime of ~4.5 ns occurred. This lifetime points to the presence of nonquenched flavin, and is also found for free FAD in 80% glycerol (unpublished observations). The largest effect on both barycentres and fractional contributions was found in the temperature range from 293 K down to 223 K, where only two lifetimes were present (Fig. 7).

A striking resemblance exists between the lifetime patterns of the tyrosine mutants in 80% glycerol and those reported for the enzyme dihydrolipoyl dehydrogenase (Bastiaens et al., 1992a,b), the spatial structure of which near the active site greatly resembles that of GR (Mattevi et al., 1991). For dihydrolipoyl dehydrogenase, a lifetime spectrum with three components at ~0.17 ns, 1.3 ns, and 2.5 ns was found in aqueous solution at pH 7, 293 K. In 80% glycerol the lifetime spectrum contained only two components, which show a temperature dependence similar to that found for the *E. coli* GR mutants at low temperatures. The lifetime values found for the tyrosine mutants of *E. coli* GR in 80% glycerol are shorter than those reported for wild-type *Azotobacter vinelandii* dihydrolipoyl dehydrogenase, but do resemble those of the more solvent-accessible deletion mutant of dihydrolipoyl dehydrogenase, which lacks 14 C-terminal amino acids (Bastiaens et al., 1992a). These results show that the large differences in the fluorescence

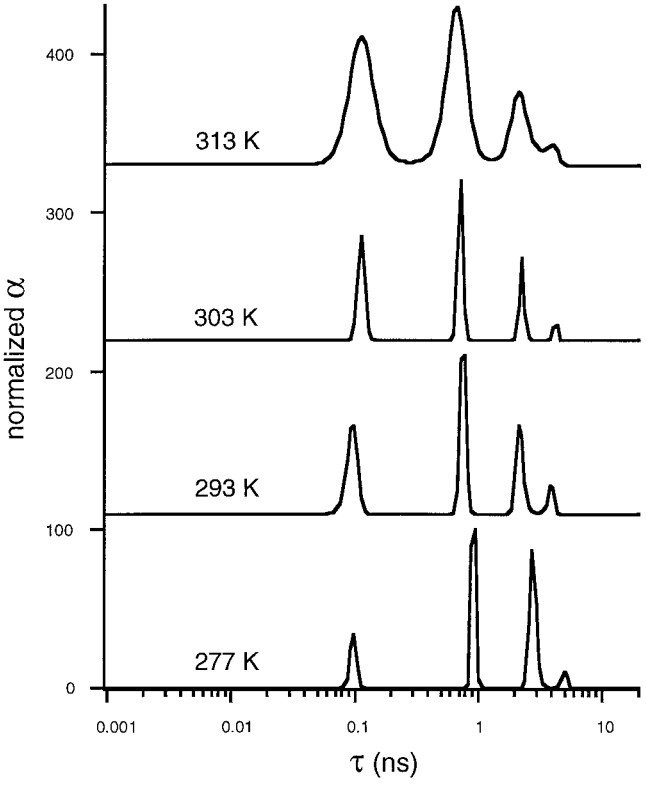

FIGURE 6 Temperature dependence of the fluorescence lifetime distribution of the mutant GR Y177G in 50 mM potassium phosphate buffer (pH 7.6). For clarity, a vertical offset has been applied.

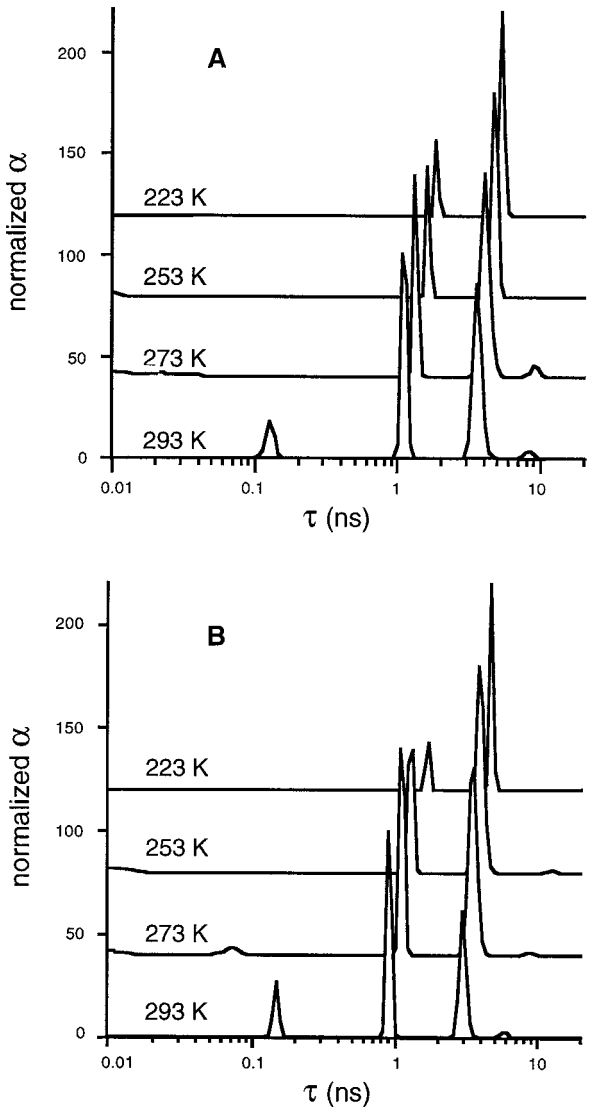

FIGURE 7 Temperature dependence of the fluorescence lifetime distributions of the mutants GR Y177F (*A*) and Y177G (*B*) in 80% glycerol, 50 mM potassium phosphate buffer (pH 7.6). For clarity, a vertical offset has been applied.

lifetime behavior of these homologous enzymes in aqueous solution are cancelled out to a large extent by varying the experimental conditions. In our opinion, this strongly favors the idea of multiple quenching sites, of which the contribution and rate of quenching strongly depend on the exact microstructure of the enzyme.

Fluorescence anisotropy decay

The flavin fluorescence anisotropy decay of wild-type *E. coli* glutathione reductase differs strongly from that of the tyrosine mutants GR Y177F and Y177G. In aqueous solution at pH 7.6 and 293 K, wild-type GR shows a distinct fluorescence depolarization (Fig. 8). This process can be described by a single rotational correlation time of 2 ns (Fig. 9). The effect of overall protein tumbling could not be

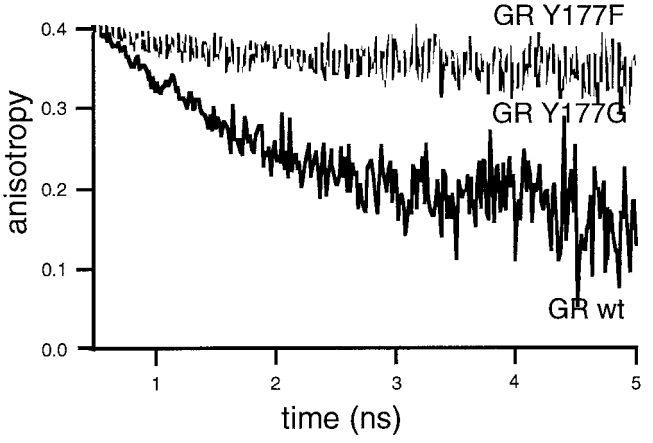

FIGURE 8 Experimental fluorescence anisotropy decay of wild-type GR and mutants Y177F and Y177G in 50 mM potassium phosphate buffer (pH 7.6) at 293 K.

resolved: based on a molecular mass of 100 kDa, one expects from the modified Stokes-Einstein relation (Visser and Lee, 1980) a rotational correlation time of 38 ns at 293 K. Owing to a much shorter fluorescence lifetime for both wild-type GR and the tyrosine mutants (27 ps and ~0.15 ns, respectively), the intensity of the signal is insufficient to resolve such a long correlation time. For this reason, only the longer lifetime components will contribute to the anisotropy decay. The rapid decay found for wild-type enzyme corresponds to the results reported for human glutathione reductase (Bastiaens et al., 1992b). In that study, a rotational correlation time of 1.5 ns at 283 K was assigned to restricted reorientational motion of the flavins. However, in the present study, neither tyrosine mutant shows this rapid depolarization: the flavin appears to be rigidly bound on the nanosecond time scale. A correlation time in the range between 2 and 7 ns was resolved, but this process only contributed with a very small amplitude. In 80% glycerol (v/v) between 293 K and 223 K, this depolarizing effect was invariably described by a correlation time of 6–7 ns. The time constant of this component agrees with a depolarizing

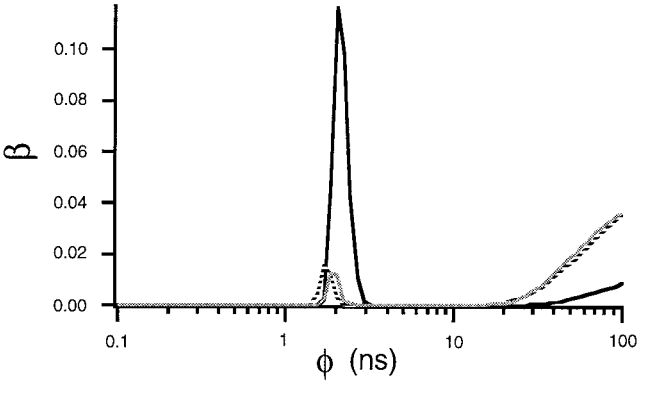

FIGURE 9 Distribution of correlation times of wild-type GR (*black solid line*) and mutants Y177F (*dashed line*) and Y177G (*gray line*) in 50 mM potassium phosphate buffer (pH 7.6) at 293 K.

process reported for human erythrocyte glutathione reductase that was assigned to intramolecular energy transfer between the two flavins (Bastiaens et al., 1992b).

Based on the fluorescence lifetime experiments described above, we find no reason to assume the flavin to be more mobile in wild-type GR than in the mutants. In fact, the fluorescence dynamics indicate a more flexible structure for the mutant enzymes. The difference between wild-type GR and the tyrosine mutants leads us to propose a novel mechanism for the fast anisotropy decay: not flavin mobility itself, but the formation of a charge transfer complex between the flavin in the excited state and Tyr^{177} may cause the rapid depolarization. From studies of binary complexes between wild-type glutathione reductase and substrate analogs, there is evidence that Y177 is indeed involved in the process of fluorescence depolarization (P. A. W. van den Berg et al., manuscript in preparation). Together with temperature and viscosity studies, these results suggest that flavin excitation of enzyme molecules with Y177 in the "out" position induces relaxation of the protein environment, followed by the formation of a charge transfer complex that is accompanied by a change in the direction of the emission dipole moment of the flavin.

DISCUSSION

Comparison of wild-type *E. coli* glutathione reductase with the mutants Y177F and Y177G by time-resolved polarized flavin fluorescence clearly identified Y177 as the residue responsible for the ultrafast quenching of flavin fluorescence in this enzyme. In various flavoproteins that contain a tyrosine residue adjacent to the flavin, the extremely low flavin fluorescence intensity of these enzymes has been attributed to (static) quenching by this tyrosine (Maeda-Yorita et al., 1991; Karplus et al., 1991; Williams, 1992). The 7-ps fluorescence lifetime component observed in this study expresses the (pseudo)dynamic nature of quenching, thereby contradicting the concept of a "dark" complex that is formed in the ground state and remains dark in the excited state. In a similar study on human glutathione reductase, Bastiaens et al. (1992b) proposed that the observed ultrafast quenching resulted from exciplex formation between the flavin and Y197. By comparing wild-type *E. coli* GR with mutants in which Y177 is replaced by either Phe or Gly, we have proved that an excited state reaction takes place. The ultrashort fluorescence lifetime and the invariance of this component with respect to temperature and viscosity indicate that the mechanism of quenching is not collisional quenching, but electron transfer from tyrosine to the light-excited flavin. Such photoinduced electron transfer could generate an extremely efficient pathway to return to the singlet ground state in a nonradiative way.

Excited-state electron transfer is known to be relevant for a variety of chemical and biological processes and has been studied extensively (for a selection, see Rehm and Weller, 1970; Marcus and Sutin, 1985; Barbara et al., 1996). An appropriate expression for the free energy of photoinduced electron transfer between a donor molecule (D) and acceptor molecule (A) in solution is given by the Rehm-Weller equation

$$\Delta G_{et} = E^{ox}_{1/2}(D/D^{+\cdot}) - E^{red}_{1/2}(A^{-\cdot}/A) - \Delta E_{0,0} - \frac{e^2}{4\pi\epsilon_0\epsilon R} \quad (5)$$

where $E^{ox}_{1/2}(D/D^{+\bullet})$ and $E^{red}_{1/2}(A^{-\bullet}/A)$ refer to the half-wave oxidation potential of the electron donor and the half-wave reduction potential of the electron acceptor, respectively. $\Delta E_{0,0}$ is the electronic excitation energy corresponding to the energy distance between vibronic ground and first excited states ($S_{0,0} \rightarrow S_{1,0}$), ϵ is the dielectric constant, ϵ_0 is the permittivity of vacuum, and R is the distance between donor and acceptor. For intramolecular photoinduced electron transfer in proteins the same equation holds, although the precise contribution of the Coulomb energy term is difficult to calculate, owing to uncertainties in donor-acceptor contact area and the local dielectric constant. However, the Coulomb energy term will only contribute in favor of the thermodynamic probability of the electron transfer process. For free FMN, the one-electron reduction potential $EFl_{ox}/EFlH^{\bullet}$ is -238 mV at pH 7, whereas that of the second half-reaction is somewhat less negative (-172 mV versus SHE at pH 7) (Draper and Ingraham, 1968). Although the precise redox properties of the flavin are controlled by the protein environment, the midpoint redox potentials reported for free flavin and proteins of the oxidoreductase family are in the same order of magnitude (Williams, 1992): e.g., for *E. coli* dihydrolipoyl dehydrogenase, the two-electron reduction potential is -264 mV at pH 7 (Wilkinson and Williams, 1979). For glutathione reductase, a midpoint potential of -235 mV at pH 7, 293 K has been reported (Williams, 1992). The slope of a plot of this potential as a function of pH changes from -52 to -39 mV/pH unit at pH 7.4, thereby indicating the presence of other dissociable groups, whose pK_a values are linked to the redox state of the enzyme (O'Donnell and Williams, 1983). A stable oxidized tyrosine radical is known to be involved in electron transfer processes in ribonucleotide reductase and the photosynthetic water-splitting system (Reichard and Ehrenberg, 1983; Barry and Babcock, 1987; Prince, 1988). The oxidation potential for the $Tyr/Tyr^{\bullet}$ couple at pH 7 in aqueous solution determined by pulse radiolysis and cyclic voltammetry is $+930$ mV (Harriman, 1987). For photosystem II, an E_{m7} of $+760$ mV has been reported for the tyrosine $D/D^{\bullet}$ couple, whereas that for the tyrosine $Z/Z^{\bullet}$ has been estimated at $E_{m7} = +1000$ mV (Boussac and Etienne, 1984). The oxidation potential of a tyrosine radical inside a protein thus seems to be in line with the value found in aqueous solution. The redox potential of the $Tyr/Tyr^{\bullet}$ couple in aqueous solution becomes less positive at increasing pH, thereby facilitating electron transfer (Harriman, 1987). Based on the redox potentials mentioned above and $R = 3.1$ Å (edge to edge distance between Y177 and N10 of the flavin), the excitation energy of 2.53 eV (490 nm) is more

than sufficient to induce electron transfer from Tyr^{177} to the isoalloxazine ring. The rate of electron transfer k_{et} is determined by the free energy, as well as the reorganization energy λ and the distance R (for relevant equations, see Marcus, 1956; Marcus and Sutin, 1985; Moser et al., 1992).

Comparison of the rates of electron transfer in a variety of biological and (semi)synthetical systems reveals typical rate constants of 1 ps^{-1} to 0.1 ps^{-1} for donor-acceptor distances of 5 Å, whereas distances of 10 Å generally result in rates between 10 ns^{-1} and 1 ns^{-1} (Moser et al., 1992). The ultrafast fluorescence quenching in *E. coli* glutathione reductase corresponds to a rate constant of ~0.14 ps^{-1} and thus agrees well with the expected rate of electron transfer in this system. Based on the known redox potentials and the excitation energy of the flavin, electron transfer is likely to be the common mechanism for fluorescence quenching in flavoproteins that contain a tyrosine residue juxtapositioned to the flavin.

A similar mechanism of flavin fluorescence quenching has been reported for tryptophan in *Desulfovibrio vulgaris* flavodoxin (Visser et al., 1987). For the $Trp/Trp^{\bullet}$ couple at pH 7 in aqueous solution, the oxidation potential is 1.015 V (Harriman, 1987). Picosecond absorption spectroscopy carried out with this enzyme revealed the existence of a transient exciplex absorption band of ~30 ps lifetime, which could be attributed to electron transfer from tryptophan to the excited flavin (Karen et al., 1987). A study on pea ferredoxin-$NADP^+$ reductase was also reported recently, in which replacement of the tyrosine adjacent to the flavin by tryptophan as well as phenylalanine resulted in a minor increase in the flavin fluorescence quantum yield, whereas nonaromatic substitutions resulted in a large increase in fluorescence (Calcaterra et al., 1995). However, in our study electron transfer did not occur in the phenylalanine mutant of *E. coli* GR, and the average fluorescence lifetime was almost the same as for the glycine mutant. A plausible explanation for this is a different orientation of the Phe side chain that is unfavorable for electron transfer to the light-excited isoalloxazine. In the crystal structure of *E. coli* GR, the closest contacts between the flavin and Y177 occur via the N10 and C9α of the flavin and the hydroxyl moiety of the tyrosine (3.1 and 3.2 Å respectively; Table 3). The hydroxyl moiety is close to the N5 and C4α of the isoalloxazine ring, which can act as electrophilic sites. In those enzymes in solution, in which Y177 occupies the "in" position, the exact site of the phenolic side chain with respect to the isoalloxazine ring may differ from that found in the crystal structures, and small positional fluctuations are likely to occur. Upon movement of the phenolic side chain toward the "out" position, the distance will only increase, and the hydroxyl moiety in particular will be turned away from the flavin.

TABLE 3 Distances between the hydroxyl oxygen atom of Y177 and the isoalloxazine ring of FAD in wild-type *E. coli* GR*

Atom	Å	Atom	Å	Atom	Å
N1	4.1	C4α	3.7	C8	4.5
C2	5.0	N5	3.7	C8M	5.7
O2	5.9	C5α	3.5	C9	3.7
N3	5.3	C6	4.3	C9α	3.2
C4	4.8	C7	4.7	C10α	3.4
O4	5.7	C7M	5.9	N10	3.1

*Distances were retrieved from the crystal structure of *E. coli* GR at 1.86-Å resolution, deposited by Mittl and Schulz (1994).

An alternative explanation for the absence of the ultrafast component in the lifetime pattern of GR Y177F is that a phenolic side chain itself is a more effective electron donor. A recent study on ribonucleotide reductase, in which long-range electron transfer occurs via a pathway that contains two tyrosine residues, showed that preservation of the aromatic character alone was not enough for electron transfer (Ekberg et al., 1996). The latter study indicated that the efficiency of electron transfer via a hydrogen bond approaches that of a covalent bond. However, based on the distances in the crystal structure and the direction of motion of the phenolic side chain upon cofactor binding, the formation of a hydrogen bond between the tyrosine and the flavin in glutathione reductase seems unlikely.

The fact that the interaction between the flavin and Y177 is perturbed in high concentrations of glycerol supports the concept that the exact position of the aromatic side chain is critical for electron transfer. Besides its function as a viscogen, glycerol is also known to influence protein structure. The cosolvent glycerol is preferentially excluded from the protein-solvent interface (Timasheff et al., 1976; Timasheff, 1993), and it can therefore induce a decrease in the volume and compressibility of the protein interior (Priev et al., 1996). Consequently, protein fluctuations can be dampened as a result of both increased friction between the peptide chain and the solvent molecules and increased friction between the amino acid residues in the protein interior itself. Gekko and Timasheff (1981a,b) have suggested that a balance between repulsion of glycerol from hydrophobic surfaces of the protein and interaction with the polar regions is, in fact, the origin of the well-known stabilizing effect of glycerol (Jarabak et al., 1966; Bradbury and Jakoby, 1972; Ogle, 1983). Moreover, glycerol can also induce structural changes in a protein (Pourplanche et al., 1994; Raibekas and Massey, 1996). Raibekas and Massey (1996) suggested that these structural changes may originate from the same repulsion mechanism, assuming the protein has solvent-exposed nonpolar residues together with flexible regions, as is often the case in the active site. In *E. coli* GR, the NADPH-binding pocket is accessible to the solvent. Although the crystal structure contains many water molecules (Mittl and Schulz, 1994), none of them are positioned directly between Y177 and the isoalloxazine ring. A large part of this binding pocket is, in principle, accessible for glycerol as well. Perturbation of the interaction between the isoalloxazine ring and Y177 as indicated by the time-resolved fluorescence and absorbance data may originate from direct interaction of this residue with glycerol molecules, but more likely is an indirect effect of glycerol on the hydration layer and, con-

sequently, the protein structure. The fact that the flavin-tyrosine interaction remained nearly intact when sucrose was used as a cosolvent can be the result of the different interaction between sucrose molecules and the solvated protein, but a decreased accessibility of the active site, resulting from the bigger size of the cosolvent, may also play a role (Yedgar et al., 1995).

Supporting evidence for a close interaction between flavin and Y177 in *E. coli* GR in solution, which is absent in GR Y177F, was found in the absorption spectra. Both fluorescence and absorption data of wild-type *E. coli* GR and mutants Y177F and Y177G are in perfect agreement with studies on *E. coli* dihydrolipoyl dehydrogenase. Maeda-Yorita et al. (1991) reported that mutation of Ile^{184}, which is at the position equivalent to that of Y177, into a tyrosine resulted in nearly complete quenching of the FAD fluorescence. A redshift of the absorption maximum of 455 nm to 462 nm, mimicking the situation in glutathione reductase, was also found. Although the authors mentioned that the spectral shape of the spectrum did not change upon introduction of the I184Y mutation, the published spectra do reveal a more pronounced shoulder at the red edge of the spectrum, similar to that described here for wild-type *E. coli* glutathione reductase. It thus seems possible to relate specific interactions, between the flavin and its microenvironment, of a nature other than the occurrence of a charge transfer interaction with the redox-active thiolate, to particular properties of the flavin absorption spectrum.

Although the juxtapositioning of a tyrosine residue and the isoalloxazine ring is a commonly observed phenomenon in flavoproteins, the biological function of this architecture is still not clear. Crystal structures of glutathione reductase as well as ferredoxin-$NADP^+$ reductase and *Crithidia fasciculata* trypanothione reductase that were complexed with their nicotinamide cofactor revealed a sandwich stacking of this cofactor between the isoalloxazine ring and the tyrosyl moiety, thereby stabilizing binding (Pai et al., 1988; Karplus and Schulz, 1989; Bailey et al., 1994; Serre et al., 1996). A similar mode of cofactor binding with a phenylalanine instead of a tyrosine was reported for NAD(P)H: quinone oxidoreductase (Li et al., 1995) and *Trypanosoma cruzi* trypanothione reductase (Zhang et al., 1996). Kinetic studies on *E. coli* GR mutants Y177F, Y177S, and Y177G, however, showed that neither the hydroxyl group nor the aromaticity of Y177 is strictly necessary for binding of the nicotinamide cofactor (Berry et al., 1989). Earlier, Rice et al. (1984) proposed a lid function for the tyrosine in glutathione reductase, to protect the reduced flavin against oxidation. Berry et al. (1989) showed that reoxidation of the flavin in the GR Y177 mutants is still rather slow. They proposed that the position of the active site in a cleft buried in the enzyme itself provides sufficient protection against unwanted oxidation. In the present study, the fluorescence lifetime patterns of the enzymes as a function of both temperature and viscosity strongly suggest that the tyrosine mutants are more sensitive to fluctuations and changes in the protein structure. Although a crystal structure of a GR Y177 mutant is not yet available, we expect that the mutation of tyrosine into phenylalanine or glycine has no significant effect on the active site structure of the free enzyme. The fluorescence lifetime patterns of GR Y177F and GR Y177G were almost identical, and under mild conditions the positions of the four longest lifetimes matched very well with those found for wild-type GR. If the mutation itself causes structural rearrangements within the active site, we would expect this to be reflected in changes in the flavin fluorescence lifetime patterns. Evidence for an increased flexibility of the protein structure of the mutant enzymes, in addition to the studies presented in this paper, was also found in the fluorescence lifetime patterns of the enzymes complexed to substrate analogues (P. A. W. van den Berg et al., manuscript in preparation). Under extreme conditions, such as 80% glycerol, a very small fraction of FAD was liberated from the free mutant enzymes. Upon long-term storage of these enzymes, a similar tendency was found. These results all point to a diminished stability of the mutant enzymes with respect to wild-type GR. Thermal and urea-induced denaturation studies on pea ferredoxin-$NADP^+$ reductase and mutants in which Y308 was replaced indicated that the presence of an electron-rich aromatic side chain adjacent to the isoalloxazine ring is essential for stabilization of the holoenzyme (Calcaterra et al., 1995). Optimal stabilization, however, was found for the tyrosine containing wild-type enzyme. It is possible that this contribution to the stability of the oxidized enzyme is a physiologically relevant characteristic of flavoproteins that have a tyrosine adjacent to the isoalloxazine ring.

The interpretation of the results in this paper differs significantly from that reported for human erythrocyte glutathione reductase (Bastiaens et al., 1992b). Recent studies that we performed on the human enzyme (P. A. W. van den Berg et al., manuscript in preparation) show that the differences in fluorescence lifetimes of the *E. coli* and human enzyme result merely from the improved time resolution of the current time-resolved fluorescence set-up. However, comparison of wild-type *E. coli* GR, with the site-directed mutants Y177F and Y177G in combination with temperature and viscosity studies, reveal new insights into both the mechanism of nonexponential fluorescence decay and the active-site dynamics of the glutathione reductase enzyme. One important difference we have already mentioned is the interpretation of the fast fluorescence depolarization in wild-type glutathione reductase. Based on comparison of wild-type *E. coli* GR with the tyrosine mutants and the information available from crystallographic studies (B-factors), we propose that not internal mobility of the flavin, but side-chain relaxation and concomitant formation of a charge transfer complex between Y177 and the light-excited flavin are responsible for the observed fluorescence depolarization. Supporting evidence for this model is obtained from studies of binary enzyme-substrate complexes of GR (P. A. W. van den Berg et al., manuscript in preparation). Here we want to focus on the interpretation of the highly nonexponential fluorescence decay in glutathione reductase.

In wild-type erythrocyte glutathione reductase, the heterogeneous fluorescence decay yielding five lifetime components has been explained by the existence of (at least) five conformational substates of the enzyme, with different orientations of tyrosine residue 197 (Bastiaens et al., 1992b). It was proposed that interconversion between the assumed substates corresponded to gross structural changes in the protein. From both this study on *E. coli* GR and the one on erythrocyte GR, it can be concluded that in the majority of the enzyme molecules in solution, the tyrosine residue is in close contact with the flavin, thereby probably blocking the active site, as evident in the crystal structures. However, in this study with the GR mutants, we found no evidence to sustain the model of conformational substates.

The fact that the mutant enzymes GR Y177F and GR Y177G still exhibit a rapid heterogeneous fluorescence decay can only be explained by the existence of quenching sites in the protein other than the juxtapositioned tyrosine. The similarity of the longer fluorescence lifetimes of the mutant enzymes with those found for wild-type GR strongly indicates that these quenching sites also contribute to a minor extent to the fluorescence decay of the wild-type enzyme. The ultrafast lifetime component originates from the interaction with Y177, but the identity of the other quenching components is still unknown. Residues that may also contribute to flavin fluorescence quenching are Phe^{161} and Phe^{344}, which are at an edge-to-edge distance of 4.3 Å and 4.8 Å, respectively, from the isoalloxazine ring. However, the π-electron system of Phe alone may be less suited for being an electron donor. Another residue that may be involved in flavin fluorescence quenching is Cys^{47}. This residue, which is part of the redox-active disulfide bridge, is at a distance of only 3.5 Å from the C4α of the flavin, and forms a charge transfer interaction with the flavin upon reduction of the enzyme. Indications that Cys^{47} indeed contributes to flavin fluorescence are found in the study of Deonarain et al. (1990), in which a 12-fold increase in flavin fluorescence was reported for glutathione reductase C47S with respect to wild-type enzyme. Besides direct quenching of Cys^{47}, the increase in fluorescence intensity may also be caused by a conformational change in the enzyme, resulting, for instance, in an unfavorable conformation of Y177 for electron transfer to the light-excited flavin. Additional support for quenching by a redox-active cysteine is found in a study on NADH peroxidase, in which mutation of Cys^{42} into an alanine or a serine residue resulted in a ~4-fold and ~10-fold increase, respectively, in fluorescence quantum yield (Parsonage and Claiborne, 1995).

The model of multiple quenching sites is also in accord with the results of the temperature and viscosity studies. The temperature invariance of the fluorescence lifetimes of wild-type glutathione reductase reflects the rigidity of the peptide chain near the active site. Perturbation of the interaction between Y177 and the flavin by glycerol decreases the quenching efficiency by tyrosine, thereby increasing the probability of fluorescence quenching caused by the other sites. The small changes in fluorescence lifetime pattern observed for the tyrosine mutants reflect the influence of small structural rearrangements. Induction of these rearrangements by glycerol at low temperatures seems to eliminate the differences in fluorescence lifetime distributions of the structurally closely related glutathione reductase and dihydrolipoyl dehydrogenase. Although this study clearly reveals the existence of multiple quenching sites, the possibility of different conformers is not ruled out. Small structural fluctuations such as the movement of Y177 are likely to contribute to the heterogeneous character of the flavin fluorescence decay. Conformational fluctuations on the (sub)nanosecond time scale as well as on longer time scales will create a heterogeneous ensemble in which the rate and probability of fluorescence quenching by a specific site are controlled by the precise microstructure and dynamics of the light-excited enzyme molecule.

We thank Prof. Dr. R. H. Schirmer, Dr. K. Becker (University of Heidelberg, Germany), Dr. P. R. E. Mittl (Novartis, Switzerland), Dr. A. A. Raibekas (University of Michigan), Dr. W. H. J. van Berkel, and Prof. Dr. C. Laane for helpful discussions and suggestions.

This work was supported by the Netherlands Foundation for Chemical Research, with financial aid from the Netherlands Organisation for Scientific Research and the E. C. Human Capital and Mobility Programme CHRX-CT93-0166 ("Flavoproteins, Structure and Activity").

REFERENCES

Alcala, J. R., E. Gratton, and F. G. Prendergast. 1987. Interpretation of fluorescence decays in proteins using continuous lifetime distributions. *Biophys. J.* 51:925–936.

Bailey, S., A. H. Fairlamb, and W. N. Hunter. 1994. Structure of trypanothione reductase from *Crithidia fasciculata* at 2.6 Å resolution; enzyme-NADP interactions at 2.8 Å resolution. *Acta Crystallogr. D.* 50: 139–154.

Bajzer, Z., and F. G. Prendergast. 1993. A model for multiexponential tryptophan fluorescence intensity decay in proteins. *Biophys. J.* 65: 2313–2323.

Barbara, P. F., T. J. Meyer, and M. A. Ratner. 1996. Contemporary issues in electron transfer research. *J. Phys. Chem.* 100:13148–13168.

Barry, B. A., and G. T. Babcock. 1987. Tyrosine radicals are involved in the photosynthetic oxygen-evolving system. *Proc. Natl. Acad. Sci. USA.* 84:7099–7103.

Bashir, A., R. N. Perham, N. S. Scrutton, and A. Berry. 1995. Altering kinetic mechanism and enzyme stability by mutagenesis of the dimer interface of glutathione reductase. *Biochem. J.* 312:527–533.

Bastiaens, P. I. H., A. van Hoek, J. A. E. Benen, J. C. Brochon, and A. J. W. G. Visser. 1992a. Conformational dynamics and intersubunit energy transfer in wild-type and mutant lipoamide dehydrogenase from *Azotobacter vinelandii*. *Biophys. J.* 63:839–853.

Bastiaens, P. I. H., A. van Hoek, W. F. Wolkers, J. C. Brochon, and A. J. W. G. Visser. 1992b. Comparison of the dynamical structures of lipoamide dehydrogenase and glutathione reductase by time-resolved polarized flavin fluorescence. *Biochemistry.* 31:7050–7060.

Beechem, J. M., and L. Brand. 1985. Time-resolved fluorescence of proteins. *Annu. Rev. Biochem.* 54:43–71.

Benen, J., W. van Berkel, Z. Zak, T. Visser, C. Veeger, and A. de Kok. 1991. Lipoamide dehydrogenase from *Azotobacter vinelandii*: site-directed mutagenesis of the His450-Glu455 diad. Spectral properties of wild type and mutated enzymes. *Eur. J. Biochem.* 202:863–872.

Berry, A., N. S. Scrutton, and R. N. Perham. 1989. Switching kinetic mechanism and putative proton donor by directed mutagenesis of glutathione reductase. *Biochemistry.* 28:1264–1269.

Bismuto, E., G. Irace, I. Sirangelo, and E. Gratton. 1996. Pressure-induced perturbation of ANS-apomyoglobin complex: frequency domain fluorescence studies on native and acidic compact states. *Protein Sci.* 5:121–126.

Boussac, A., and A. L. Etienne. 1984. Midpoint potential of signal II (slow) in tris-washed photosystem-II particles. *Biochim. Biophys. Acta.* 766: 576–581.

Bradbury, S. L., and W. B. Jakoby. 1972. Glycerol as an enzyme-stabilizing agent: effects on aldehyde dehydrogenase. *Proc. Natl. Acad. Sci. USA.* 69:2373–2376.

Brochon, J. C. 1994. Maximum entropy method of data analysis in time-resolved spectroscopy. *Methods Enzymol.* 240:262–311.

Calcaterra, N. B., G. A. Picó, E. G. Orellano, J. Ottado, N. Carrillo, and E. A. Ceccarelli. 1995. Contribution of the FAD binding site residue tyrosine 308 to the stability of pea ferredoxin-$NADP^+$ oxidoreductase. *Biochemistry.* 34:12842–12848.

Careri, G., P. Fasella, and E. Gratton. 1979. Enzyme dynamics: the statistical physics approach. *Annu. Rev. Biophys. Bioeng.* 8:69–97.

Deonarain, M. P., A. Berry, N. S. Scrutton, and R. N. Perham. 1989. Directed mutagenesis of the redox-active disulphide bridge in glutathione reductase from *Escherichia coli. Biochemistry.* 28:9602–9607.

Deonarain, M. P., N. S. Scrutton, A. Berry, and R. N. Perham. 1990. Alternative proton donors/acceptors in the catalytic mechanism of the glutathione reductase of *Escherichia coli*: the role of histidine-439 and tyrosine-99. *Proc. R. Soc. Lond. B.* 241:179–186.

Draper, R. D., and L. L. Ingraham. 1968. A potentiometric study of the flavin semiquinone equilibrium. *Arch. Biochem. Biophys.* 125:802–808.

Ekberg, M., M. Sahlin, M. Eriksson, and B.-M. Sjöberg. 1996. Two conserved tyrosine residues in protein R1 participate in an intermolecular electron transfer in ribonucleotide reductase. *J. Biol. Chem.* 271: 20655–20659.

Ermler, U., and G. E. Schulz. 1991. The three-dimensional structure of glutathione reductase from *Escherichia coli* at 3.0 Å resolution. *Proteins Struct. Funct. Genet.* 9:174–179.

Ferreira, S. T., L. Stella, and E. Gratton. 1994. Conformational dynamics of bovine Cu, Zn superoxide dismutase revealed by time-resolved fluorescence spectroscopy of the single tyrosine residue. *Biophys. J.* 66: 1185–1196.

Frauenfelder, H., and E. Gratton. 1986. Protein dynamics and hydration. *Methods Enzymol.* 127:207–216.

Frauenfelder, H., F. Parak, and R. D. Young. 1988. Conformational substates in proteins. *Annu. Rev. Biophys. Biophys. Chem.* 17:451–479.

Frauenfelder, H., S. G. Sligar, and P. G. Wolynes. 1991. The energy landscapes and motions of proteins. *Science.* 254:1598–1603.

Gekko, K., and S. N. Timasheff. 1981a. Mechanism of protein stabilization by glycerol: preferential hydration in glycerol-water mixtures. *Biochemistry.* 20:4667–4676.

Gekko, K., and S. N. Timasheff. 1981b. Thermodynamic and kinetic examination of protein stabilization by glycerol. *Biochemistry.* 20: 4677–4686.

Greer, S., and R. N. Perham. 1986. Glutathione reductase from *Escherichia coli*: cloning and sequence analysis of the gene and relationship to other flavoprotein disulfide oxidoreductases. *Biochemistry.* 25:2736–2742.

Harriman, A. 1987. Further comments on the redox potentials of tryptophan and tyrosine. *J. Phys. Chem.* 91:6102–6104.

Holmgren, A. 1985. Thioredoxin. *Annu. Rev. Biochem.* 54:237–271.

Jarabak, J., A. E. Seeds, Jr., and P. Talalay. 1966. Reversible cold inactivation of a 17β-hydroxysteroid dehydrogenase of human placenta: protective effect of glycerol. *Biochemistry.* 5:1269–1279.

Karen, A., M. T. Sawada, F. Tanaka, and N. Mataga. 1987. Dynamics of excited flavoproteins-picosecond laser photolysis studies. *Photochem. Photobiol.* 45:49–53.

Karplus, P. A., M. J. Daniels, and J. R. Herriott. 1991. Atomic structure of ferredoxin-$NADP^+$-reductase: prototype for a structurally novel flavoenzyme family. *Science.* 251:60–66.

Karplus, M., and J. A. McCammon. 1983. Dynamics of proteins: elements and function. *Annu. Rev. Biochem.* 53:263–300.

Karplus, P. A., and G. E. Schulz. 1987. Refined structure of glutathione reductase at 1.54 Å resolution. *J. Mol. Biol.* 195:701–729.

Karplus, P. A., and G. E. Schulz. 1989. Substrate binding and catalysis by glutathione reductase as derived from refined enzyme:substrate crystal structures at 2 Å resolution. *J. Mol. Biol.* 210:163–180.

Li, R., M. A. Bianchet, P. Talalay, and L. M. Amzel. 1995. The three-dimensional structure of NAD(P)H:quinone reductase, a flavoprotein involved in cancer chemoprotection and chemotherapy: mechanism of the two-electron reduction. *Proc. Natl. Acad. Sci. USA.* 92:8846–8850.

Livesey, A. K., and J. C. Brochon. 1987. Analyzing the distribution of decay constants in pulse-fluorimetry using the maximum entropy method. *Biophys. J.* 52:693–706.

Maeda-Yorita, K., G. C. Russell, J. R. Guest, V. Massey, and C. H. Williams, Jr. 1991. Properties of lipoamide dehydrogenase altered by site-directed mutagenesis at a key residue (I184Y) in the pyridine nucleotide binding domain. *Biochemistry.* 30:11788–11795.

Marcus, R. A. 1956. On the theory of oxidation-reduction reactions involving electron transfer. *J. Chem. Phys.* 24:966–978.

Marcus, R. A., and N. Sutin. 1985. Electron transfers in chemistry and biology. *Biochim. Biophys. Acta.* 811:265–322.

Mattevi, A., A. J. Schierbeek, and W. G. Hol. 1991. Refined crystal structure of lipoamide dehydrogenase from *Azotobacter vinelandii* at 2.2 Å resolution. A comparison with the structure of glutathione reductase. *J. Mol. Biol.* 220:975–994.

Mérola, F., R. Rigler, A. Holmgren, and J. C. Brochon. 1989. Picosecond tryptophan fluorescence of thioredoxin: evidence for discrete species in slow exchange. *Biochemistry.* 28:3383–3398.

Millar, D. P. 1996. Time-resolved fluorescence spectroscopy. *Curr. Opin. Struct. Biol.* 6:637–642.

Mittl, P. R. E., and G. E. Schulz. 1994. Structure of glutathione reductase from *Escherichia coli* at 1.86 Å resolution: comparison with the enzyme from human erythrocytes. *Protein Sci.* 3:799–809.

Moser, C. C., J. M. Keske, K. Warncke, R. S. Farid, and P. L. Dutton. 1992. Nature of biological electron transfer. *Nature.* 355:796–802.

O'Connor, D. V., and D. Phillips. 1984. Time-Correlated Single Photon Counting. Academic Press, London.

O'Donnell, M. E., and C. H. Williams, Jr. 1983. Proton stoichiometry in the reduction of the FAD and disulfide of *Escherichia coli* thioredoxin reductase. *J. Biol. Chem.* 258:13795–13805.

Ogle, T. F. 1983. Action of glycerol and sodium molybdate in stabilization of the progesterone receptor from rat trophoblast. *J. Biol. Chem.* 258: 4982–4988.

Pai, E. F., P. A. Karplus, and G. E. Schulz. 1988. Crystallographic analysis of the binding of NADPH, NADPH fragments, and NADPH analogues to glutathione reductase. *Biochemistry.* 27:4465–4474.

Pai, E. F., and G. E. Schulz. 1983. The catalytic mechanism of glutathione reductase as derived from x-ray diffraction analyses of reaction intermediates. *J. Biol. Chem.* 258:1752–1757.

Parsonage, D., and A. Claiborne. 1995. Analysis of the kinetic and redox properties of NADH peroxidase C42S and C42A mutants lacking the cysteine- sulfenic acid redox center. *Biochemistry.* 34:435–441.

Pourplanche, C., C. Lambert, M. Berjot, J. Marx, C. Chopard, A. J. P. Alix, and V. Larreta-Garde. 1994. Conformational changes of lipoxygenase (LOX) in modified environments. *J. Biol. Chem.* 269:31585–31591.

Priev, A., A. Almagor, S. Yedgar, and B. Gavish. 1996. Glycerol decreases the volume and compressibility of protein interior. *Biochemistry.* 35: 2061–2066.

Prince, R. C. 1988. Tyrosine radicals. *Trends Biochem. Sci.* 13:286–288.

Raibekas, A. A., and V. Massey. 1996. Glycerol-induced development of catalytically active conformation of *Crotalus adamanteus* L-amino acid oxidase *in vitro. Proc. Natl. Acad. Sci. USA.* 93:7546–7551.

Rehm, D., and A. Weller. 1970. Kinetics of fluorescence quenching by electron and H-atom transfer. *Israel J. Chem.* 8:259–271.

Reichard, P., and A. Ehrenberg. 1983. Ribonucleotide reductase—a radical enzyme. *Science.* 221:514–519.

Rice, D. W., G. E. Schulz, and J. R. Guest. 1984. Structural relationship between glutathione reductase and lipoamide dehydrogenase. *J. Mol. Biol.* 174:483–496.

Scrutton, N. S., A. Berry, and R. N. Perham. 1987. Purification and characterization of glutathione reductase encoded by a cloned and overexpressed gene in *Escherichia coli*. *Biochem. J.* 245:875–880.

Scrutton, N. S., A. Berry, and R. N. Perham. 1990. Redesign of the coenzyme specificity of a dehydrogenase by protein engineering. *Nature.* 343:38–43.

Serre, L., F. M. D. Vellieux, M. Medina, C. Gomez-Moreno, J. C. Fontecilla-Camps, and M. Frey. 1996. X-ray structure of the ferredoxin: $NADP^+$ reductase from the cyanobacterium *Anabaena* PCC 7119 at 1.8 Å resolution, and crystallographic studies of $NADP^+$ binding at 2.25 Å resolution. *J. Mol. Biol.* 263:20–39.

Siemiarczuk, A., B. D. Wagner, and W. R. Ware. 1990. Comparison of the maximum entropy and exponential series methods for the recovery of distributions of lifetimes from fluorescence lifetime data. *J. Phys. Chem.* 94:1661–1666.

Thieme, R., E. F. Pai, R. H. Schirmer, and G. E. Schulz. 1981. Three-dimensional structure of glutathione reductase at 2 Å resolution. *J. Mol. Biol.* 152:763–782.

Timasheff, S. N. 1993. The control of protein stability and association by weak interactions with water: how do solvents affect these processes? *Annu. Rev. Biophys. Biomol. Struct.* 22:67–97.

Timasheff, S. N., J. C. Lee, E. P. Pittz, and N. Tweedy. 1976. The interaction of tubulin and other proteins with structure-stabilizing solvents. *J. Colloid Interface Sci.* 55:658–663.

van Hoek, A., and A. J. W. G. Visser. 1985. Artefact and distortion sources in time correlated single photon counting. *Anal. Instrum.* 14:359–378.

van Hoek, A., and A. J. W. G. Visser. 1992. Cw dye laser synchronously pumped by the third harmonic of a mode-locked cw Nd:YLF laser for tunable blue and green excitation and picosecond time-correlated photon counting detection. *Proc. SPIE.* 1640:325–329.

van Hoek, A., K. Vos, and A. J. W. G. Visser. 1987. Ultrasensitive time-resolved polarized fluorescence spectroscopy as a tool in biology and medicine. *IEEE J. Quantum Electron.* QE-23:1812–1820.

Visser, A. J. W. G., and J. Lee. 1980. Lumazine protein from the bioluminescent bacterium *Photobacterium phosphoreum.* A fluorescence study of the protein-ligand equilibrium. *Biochemistry.* 19:4366–4372.

Visser, A. J. W. G., A. van Hoek, T. Kulinski, and J. Le Gall. 1987. Time-resolved fluorescence studies of flavodoxin. Demonstration of picosecond fluorescence lifetimes of FMN in *Desulfovibrio* flavodoxins. *FEBS Lett.* 224:406–410.

Vos, K., A. van Hoek, and A. J. W. G. Visser. 1987. Application of a reference convolution method to tryptophan fluorescence in proteins. A refined description of rotational dynamics. *Eur. J. Biochem.* 165:55–63.

Welch, G. R., B. Somogyi, and S. Damjanovich. 1982. The role of protein fluctuations in enzyme action: a review. *Prog. Biophys. Mol. Biol.* 39:109–146.

Wilkinson, K. D., and C. H. Williams, Jr. 1979. Evidence for multiple electronic forms of two-electron-reduced lipoamide dehydrogenase from *Escherichia coli*. *J. Biol. Chem.* 254:852–862.

Williams, C. H., Jr. 1976. Flavin-containing dehydrogenases. *In* The Enzymes, 3rd Ed., Vol. 13. P. D. Boyer, editor. Academic Press, New York. 89–173.

Williams, C. H., Jr. 1992. Lipoamide dehydrogenase, glutathione reductase, thioredoxin reductase, and mercuric ion reductase—a family of flavoenzyme transhydrogenases. *In* Chemistry and Biochemistry of Flavoenzymes, Vol 3. F. Müller, editor. CRC Press, Boca Raton, FL. 121–213.

Yedgar, S., C. Tetreau, B. Gavish, and D. Lavalette. 1995. Viscosity dependence of O_2 escape from respiratory proteins as a function of cosolvent molecular weight. *Biophys. J.* 68:665–670.

Zhang, Y., C. S. Bond, S. Bailey, M. L. Cunningham, A. H. Fairlamb, and W. N. Hunter. 1996. The crystal structure of trypanothione reductase from the human pathogen *Trypanosoma cruzi* at 2.3 Å resolution. *Protein Sci.* 5:52–61.