Graddis, T. J., Klig, L. S., Yanofsky, C., & Oxender, D. L. (1988) *Proteins: Struct., Funct., Genet. 4*, 173-181.

Guyader, M., Emerman, M., Sonigo, P., Clavel, F., Montaigner, L., & Alizon, M. (1987) *Nature 326*, 662-669.

Herskowitz, I. (1987) *Nature 329*, 219-222.

Katoh, I., Ikawa, Y., & Yoshinaka, Y. (1989) *J. Virol. 63*, 2226-2232.

Kohl, N. E., Emini, E. A., Schleif, W. A., Davis, L. J., Heimbach, J. C., Dixon, R. A. F., Scolnick, E. M., & Sigal, I. S. (1988) *Proc. Natl. Acad. Sci. U.S.A. 85*, 4686-4690.

Lapatto, R., Blundell, T., Hemmings, A., Overington, J., Wilderspin, A., Wood, S., Merson, J. R., Whittle, P. J., Danley, D. E., Geoghegan, K. F., Hawlylik, S. J., Lee, S. E., Scheld, K., & Hobart, P. M. (1989) *Nature 342*, 299-302.

Lightfoote, M. M., Coligan, J. E., Folks, T. M., Fauci, A. S., Martin, M. A., & Venkatesan, S. (1986) *J. Virol. 60*, 771-775.

Lillehoj, E. P., Salazar, F. H. R., Mervis, R. J., Raum, M. G., Chan, H. W., Ahmad, N., & Venkatesan, S. (1988) *J. Virol. 62*, 3053-3058.

Malim, M. H., Böhnlein, S., Hauber, J., & Cullen, B. R. (1989) *Cell 58*, 205-214.

McQuade, T. J., Tomasselli, A. G., Liu, L., Karacostas, V., Moss, B., Sawyer, T. K., Heinrikson, R. L., & Tarpley, W. G. (1990) *Science 247*, 454-456.

Meek, T. D., Dayton, B. D., Metcalf, B. W., Dreyer, G. B., Strickler, J. E., Gorniak, J. G., Rosenberg, M., Moore, M. L., Magaard, V. W., & Debouck, C. (1989) *Proc. Natl. Acad. Sci. U.S.A. 86*, 1841-1845.

Meek, T. D., Lambert, D. M., Dreyer, G. B., Carr, T. J., Tomazek, T. A., Moore, M. L., Strickler, J. E., Debouck, C., Hyland, L. J., Mathews, T. J., Metcalf, B. W., & Petteway, S. R. (1990) *Nature 343*, 90-92.

Miller, M., Jaskolski, M., Rao, J. K. M., Leis, J., & Wlodawer, A. (1989) *Nature 337*, 576-579.

Navia, M. A., Fitzgerald, P. M. D., McKeever, B. M., Leu, C.-T., Heimbach, J. C., Herber, W. K., Sigal, I. S., Darke, P. L., & Springer, J. P. (1989) *Nature 337*, 615-620.

Pearl, L. H., & Taylor, W. R. (1987) *Nature 329*, 351-354.

Pichuantes, S., Babé, L. M., Barr, P. J., & Craik, C. S. (1989) *Proteins: Struct., Funct., Genet. 6*, 324-337.

Pichuantes, S., Babé, L. M., Barr, P. J., DeCamp, D. L., & Craik, C. S. (1990) *J. Biol. Chem. 265*, 13890-13898.

Sanchez-Pescador, R., Power, M. D., Barr, P. J., Steimer, K. S., Stempien, M. M., Brown-Shimer, S. L., Gee, W. W., Renard, A., Randolph, A., Levy, J. A., Dina, D., & Luciw, P. A. (1985) *Science 227*, 484-492.

Tomasselli, A. G., Hiu, J. O., Sawyer, T. K., Staples, D. J., FitzGerald, D. J., Chaudhary, V. K., Pastan, I., & Heinrikson, R. L. (1990a) *J. Biol. Chem. 265*, 408-413.

Tomasselli, A. G., Olsen, M. K., Hui, J. O., Staples, D. J., Sawyer, T. K., Heinrikson, R. L., & Tomich, C.-S. (1990b) *Biochemistry 29*, 264-269.

Weber, I. T., Miller, M., Jaskolski, M., Leis, J., Skalka, A. M., & Wlodawer, A. (1989) *Science 243*, 928-931.

Wlodawer, A., Miller, M., Kaskolski, M., Sathyanarayana, B. K., Baldwin, E., Weber, I. T., Selk, L. M., Clawson, L., Schneider, J., & Kent, S. B. H. (1989) *Science 245*, 616-621.

Electronic Energy Transfer and Fluorescence Quenching in the Active Sites of Mercuric Reductase[†]

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ABSTRACT: The FAD-containing enzyme mercuric reductase has been studied by means of steady-state and time-resolved fluorescence spectroscopy. The fluorescence relaxation of the excited state of the isoalloxazine ring of FAD can be described by a sum of two exponential functions. The two lifetimes are not due to a different lifetime of each of the two FAD molecules of mercuric reductase. The FAD molecules are quenched dynamically by a quencher that is not sensitive to the solvent viscosity. In vitro activation induces a dynamic quenching of fluorescence, while upon binding of NADP$^+$ the FAD molecules are both statically and dynamically quenched. Time-resolved fluorescence anisotropy experiments of mercuric reductase in water show that the isoalloxazine ring probably undergoes a rapid and restricted vibrational motion of small amplitude. Electronic energy transfer occurs between the two FAD molecules at a rate of about 3.4×10^7 s^{-1}. The angle between the emission transition dipole of the donor and the absorption transition dipole of the acceptor is 137 ± 2° (or 43 ± 2°). From previous X-ray data of glutathione reductase we find that the corresponding angle is 160°. This suggests that the isoalloxazine rings of mercuric reductase and glutathione reductase are mutually tilted in slightly different ways.

Resistance to inorganic mercury in many bacteria is mediated via the flavoenzyme mercuric reductase (MR)[1] that catalyzes a two-electron reduction of mercuric ions to elemental mercury using NADPH as the electron donor. MR is a dimer with two identical subunits. Each subunit contains an FAD molecule in close contact with a redox-active disulfide group. The active enzyme has many active-site features in common with a family of disulfide oxidoreductases including

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[1] Abbreviations: MR, mercuric reductase; GR, glutathione reductase; FWHM, full width at half-maximum.

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glutathione reductase, lipoamide dehydrogenase, and trypanothione reductase (Williams, 1976; Shames et al., 1986).

The three-dimensional structures of human erythrocyte glutathione reductase (GR) and lipoamide dehydrogenase from *Azotobacter vinelandii* have been determined at a resolution of 1.54 and 2.8 Å, respectively (Karplus & Schulz, 1987; Schierbeek et al., 1989). The strong homology between MR and the two structurally characterized oxidoreductases, especially in the active-site region, suggests that the active-site tertiary structures are similar in the three enzymes (Brown et al., 1983; Carothers et al., 1989; Westphal & de Kok, 1988).

Studies of the fluorescence properties of the oxidized forms of human erythrocyte GR and lipoamide dehydrogenase from *Escherichia coli* have shown that the fluorescence quantum yield of GR is very low as compared to that of lipoamide dehydrogenase (de Kok & Visser, 1987). This was ascribed to dynamic quenching in GR. Furthermore, de Kok and Visser (1987) concluded that no energy transfer occurs between the flavins in the two subunits of the enzyme and that the flexibilities of the flavins in both enzymes are different.

In this paper we have measured the steady-state and time-resolved fluorescence of MR from the Gram-negative bacterium *Pseudomonas aeruginosa*. The study aims at investigating different rotational motions and quenching of the intrinsic isoalloxazine fluorophores of FAD. This gives information on the dynamics of the protein, which is of interest when trying to understand the catalytic mechanism. Furthermore, the possibility of electronic energy transfer between the two FAD molecules of MR is elucidated, which gives information about the mutual orientation of their isoalloxazine rings.

Materials and Methods

Enzyme and Chemicals. Mercuric reductase from *P. aeruginosa* strain PAO 9501 containing the plasmid pVS1 was purified as described previously (Sahlman & Lindskog, 1983). The purified enzyme was dialyzed against 2 M KBr followed by 50 mM potassium phosphate buffer, pH 7.3, to remove enzyme-bound NADP$^+$ (Fox & Walsh, 1982). The enzyme is present in the above-mentioned buffer or in a mixture of 60% w/w glycerol and 40% w/w buffer. Glycerol (Omnisolv, BDH, spectroscopic grade) was used as received. In order to obtain the active form of MR, the enzyme was incubated with cysteine, as previously described (Sandström & Lindskog, 1988). The enzyme concentrations were determined spectrophotometrically at 455 nm where the molar absorptivity of the enzyme bound FAD is 1.13×10^4 M^{-1} cm^{-1} (Fox & Walsh, 1982). Enzyme concentrations are given as concentrations of enzyme-bound FAD throughout this paper. NADP$^+$ concentrations were estimated spectrophotometrically at 260 nm by using a molar absorptivity of 1.76×10^4 M^{-1} cm^{-1} according to the manufacturer's specification. Free FAD was obtained by precipitating MR with acetone. NADP$^+$ and L-cysteine were purchased from Sigma.

Synthesis of ^{14}C-Labeled NADP$^+$. Labeled NADP$^+$ was synthesized from nicotinamide [U-^{14}C]adenine dinucleotide (Amersham) as described by Wang et al. (1954) except that 120 mM Tris-HCl buffer, pH 7.7, was used. The labeled NADP$^+$ was purified by ion-exchange chromatography on a Dowex 1X4-400 (Sigma) column according to Bernofsky (1980). After sample application, the column was eluted with 75 mL of water followed by a linear ammonium formate–formic acid gradient, beginning with water and ending with 1 M ammonium formate in 0.2 M formic acid. The absorbance at 260 nm was continually monitored and absorbing fractions were tested by the isocitrate assay (described below). NADP$^+$-containing fractions were pooled and lyophilized.

The amount of radiolabeled NADP$^+$ was estimated by using pig heart isocitrate dehydrogenase that quantitatively converts NADP$^+$ to NADPH. The reaction mixture contained 1.25 mM DL-isocitrate and 0.26 mg of isocitrate dehydrogenase in 3.0 mL of the above-mentioned Tris buffer. The assay was started by adding 40 μL of the sample. The absorbance increase at 340 nm was followed until it reached a constant value. The absorbance change, after correction for the absorbance of the sample at 340 nm, was used to determine the NADP$^+$ concentration.

Equilibrium Dialysis. Five dialysis cells were filled with 2.0 mL of 5 μM mercuric reductase in one of the cell compartments and 2.0 mL of 3, 6, 10, 25, and 50 μM ^{14}C-labeled NADP$^+$ solution to the other chamber. In order to determine the time required for NADP$^+$ equilibration, one control experiment without enzyme was carried out as described above. The cells were allowed to equilibrate at 4 °C. When the radioactivity was equally distributed in the control cell the radioactivity was measured in a Rackbeta 1218 liquid scintillation counter (LKB Wallac, Sweden).

The "clipped" enzyme, lacking 85 amino acid residues at the N-terminus, was obtained by limited proteolytic cleavage with chymotrypsin (Fox & Walsh, 1983).

Fluorescence and Absorption Measurements. Absorption spectra were recorded on a Cary 219 spectrophotometer (Varian, USA). Fluorescence spectra and steady-state anisotropies were obtained with a SPEX Fluorolog 112 (SPEX Ind., NJ), supplemented with Glan-Thompson polarizers. A standard lamp was used to calibrate the wavelength-dependent sensitivity of the instrument. The fluorescence spectra presented have thereby been corrected.

The fluorescence quantum yields were measured with rhodamine 6G as a reference ($\Phi = 0.95$; Kubin & Fletcher, 1982; Drexhage, 1977).

The emission anisotropy was calculated from

$$r_s = \frac{F_{VV} - GF_{VH}}{F_{VV} + 2GF_{VH}} \qquad (1)$$

where the subscripts refer to vertical or horizontal settings of the excitation and emission polarizers, respectively. The correction factor $G = F_{HV}/F_{HH}$ compensates for the different transmission efficiencies of vertically and horizontally polarized light. The excitation anisotropy was calculated in an analogous way. F_{VH} and F_{HV} are then interchanged in the above expressions. The limiting anisotropy, r_0, of FAD was determined by using 1,2-propandiol (Merck, analytical grade) as solvent.

Time-Resolved Measurements. A PRA 3000 system (Photophysical Research Associates, Inc., Canada) was used for single photon counting measurements. The excitation source was a thyratron-gated flash lamp (Model 510C, PRA) filled with deuterium gas and operated at about 30 kHz. The excitation wavelength was selected with an interference filter centered at 450 nm (FWHM = 10.7 nm). The emission was generally monitored above 520 nm through a longpass filter (Schott KV 520). The emission was also observed at 500 and 550 nm through interference filters (FWHM = 12.3 and 9.4 nm, respectively) for checking the wavelength dependence. The fluorescence decay $F_{VM}(t)$ was deconvoluted as a sum of exponential functions. The subscript M indicates that the emission polarizer is rotated at the so-called magic angle, i.e., 54.7° from the vertical position. The anisotropy decay $r(t)$ was deconvoluted from the sum decay

$$S(t) = F_{VV}(t) + 2KF_{HV}(t) \qquad (2)$$

and the difference decay

$$D(t) = F_{VV}(t) - KF_{HV}(t) \tag{3}$$

The correction factor K was obtained by normalizing the total number of counts F_{VV} and F_{HV} collected in $F_{VV}(t)$ and $F_{HV}(t)$, respectively, to the steady-state anisotropy r_s as

$$K = \frac{(1 - r_s)F_{VV}}{(1 + 2r_s)F_{HV}} \tag{4}$$

In all fluorescence experiments the maximum absorbance was kept below 0.1 to avoid reabsorption.

The Radiative Lifetime. τ_0 of FAD bound to mercuric reductase was calculated from the absorption spectrum according to the modified Strickler-Berg equation (Birks, 1970):

$$\frac{1}{\tau_0} = (2.88 \times 10^{-9})n^2 \frac{\int F(\nu)\,d\nu}{\int F(\nu)\nu^{-3}\,d\nu} \int \epsilon(\nu)\nu^{-1}\,d\nu \tag{5}$$

where $F(\nu)$ is the fluorescence spectrum and $\epsilon(\nu)$ is the molar absorptivity of the first electronic transition. ν denotes the wavenumber. The absorption band of the first electronic transition was separated from that of the second electronic transition by the method of Valeur and Weber (1977). It is then assumed that the excitation anisotropy is composed of two contributions in the region 350–400 nm.

Energy-Transfer Parameters. The rate of energy transfer (k_{DA}) between two weakly dipole-coupled chromophores (an excited donor D and a ground-state acceptor A) reads (Förster, 1948, 1965)

$$k_{DA} = \frac{9000\kappa^2(\ln 10)}{128\pi^5 n^4 N_A \tau_0 R^6} J \tag{6}$$

Here n is the refractive index, R is the distance between the chromophores, and J is the spectral overlap integral:

$$J = \frac{\int F_D(\nu)\epsilon_A(\nu)\nu^{-4}\,d\nu}{\int F_D(\nu)\,d\nu} \tag{7}$$

Here $F_D(\nu)$ is the fluorescence spectrum of the donor and $\epsilon_A(\nu)$ is the molar absorptivity of the acceptor. If the spectra are given on a wavelength scale λ, J reads

$$J = \frac{\int F_D(\lambda)\epsilon_A(\lambda)\lambda^2\,d\lambda}{\int F_D(\lambda)\lambda^{-2}\,d\lambda} \tag{8}$$

In several biological and biochemical journals J has been given incorrectly as

$$J = \frac{\int F_D(\lambda)\epsilon_A(\lambda)\lambda^4\,d\lambda}{\int F_D(\lambda)\,d\lambda} \tag{9}$$

Equation 9 underestimates J when the overlap only occurs on the blue side of the fluorescence band.

In eq 6, κ^2 denotes the angular part of dipole–dipole interaction, i.e.

$$\kappa^2 = [\hat{a}\hat{d} - 3(\hat{a}\hat{r})(\hat{r}\hat{d})]^2 \tag{10}$$

κ^2 varies between 0 and 4 depending on the relative orientations of the interacting absorption ($\hat{a}$) and emission ($\hat{d}$) dipoles and the direction of their intermolecular distance vector $\hat{r}$. Here $\hat{a}$, $\hat{d}$, and $\hat{r}$ are unit vectors. For a pair of chromophores bound to a biopolymer it is usually not possible to ascertain a definite value of κ^2. However, from knowledge of the orientational freedom of the chromophores it is possible to estimate the upper and lower limits of κ^2 (Dale et al., 1979). Dale et al. (1979) have calculated these limits, assuming that the rate of reorientation of chromophores was much faster than the rate of energy transfer.

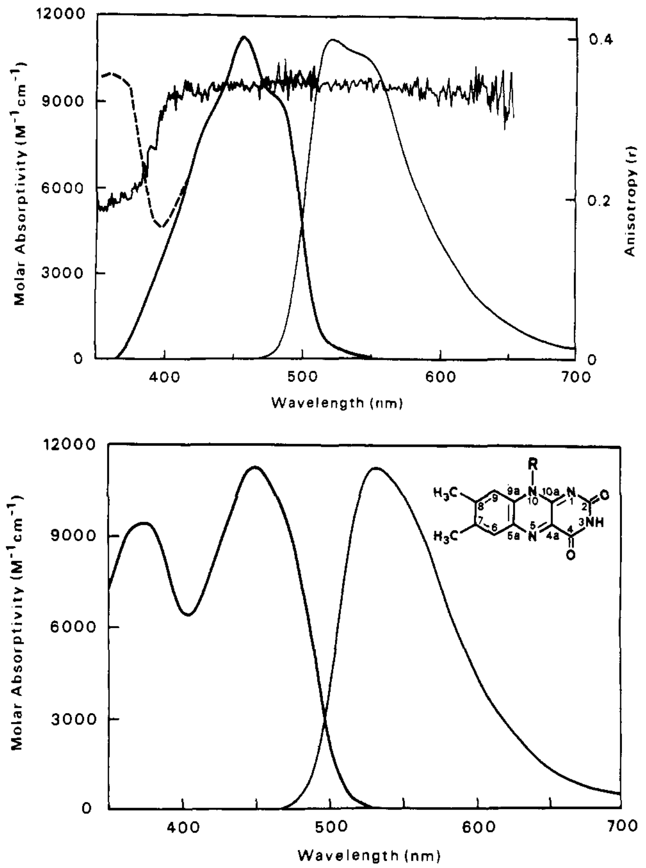

FIGURE 1: Absorption (—) and the corrected fluorescence (—) spectra of FAD bound to MR (A, top) and free FAD (B, bottom). The first electronic transition of FAD bound to MR (—) is resolved from the absorption spectra (---). The excitation (thick noisy line) and emission (thin noisy line) anisotropies of FAD bound to MR (at 298 K) are also shown in A.

RESULTS AND DISCUSSION

Spectral Data. The absorption and fluorescence spectra of mercuric reductase and FAD are presented in Figure 1, together with the fluorescence anisotropy of MR. The protein induces small spectral changes, most importantly as shoulders at about 480 and 540 nm in the absorption and the emission spectra, respectively. These changes could be due to a different binding of the two flavins to the apoprotein. We have, therefore, compared the shape of the emission spectra when exciting at different wavelengths, but no significant differences were observed. A radiative lifetime of FAD in MR was calculated to be 15.9 ns by using the resolved absorption spectrum and eq 5. The overlap integral $J = 4.60 \times 10^{-15}$ cm^3 M^{-1} was calculated from eq 8.

Fluorescence Lifetimes and Quantum Yields. The fluorescence lifetimes, the quantum yields, and the ratio between the observed average lifetime ($\langle\tau\rangle$) and the radiative lifetime (τ_0) of MR in different systems are presented in Table I. The fluorescence decay is not a single exponential function, but a sum of two exponential functions with different lifetimes yields an acceptable fit. No wavelength dependence of the fluorescence decay could be detected.

It is known that the enzyme loses about 80 amino acids partially at the N-terminus in preparations and in the cell (Fox

Table I: Fluorescence Lifetimes ($\langle\tau\rangle$, τ_1, and τ_2) and Quantum Yields (Φ_{rel} and Φ) of Mercuric Reductase in Buffer Solution (native) in a Glycerol–Buffer Mixture (glyc), in an Activated State (active), with NADP$^+$ in a Glycerol–Buffer Mixture (N/glyc), with NADP$^+$ (NADP), and of FAD in Buffer Solution[a]

MR	T (K)	α_1	τ_1 (ns)	α_2	τ_2 (ns)	$\langle\tau\rangle$ (ns)	n	$\langle\tau\rangle/\tau_0$	Φ_{rel}	Φ
native	298	0.329 (0.08)	1.47 (0.2)	0.671 (0.08)	2.75 (0.07)	2.27 (0.03)	16	0.14	1	0.12
	274	0.361 (0.06)	1.92 (0.3)	0.639 (0.06)	3.81 (0.1)	3.14 (0.1)	13	0.20	1.47	0.18
glyc	298	0.683 (0.04)	1.69 (0.07)	0.317 (0.04)	3.11 (0.09)	2.14 (0.02)	6	0.13	0.98	0.12
	274	0.506 (0.08)	2.24 (0.3)	0.494 (0.08)	4.06 (0.15)	3.15 (0.07)	6	0.19	1.42	0.17
	239	0.401 (0.08)	2.48).3)	0.599 (0.08)	5.50 (0.2)	4.30 (0.09)	4	0.27	1.90	0.23
active	298	0.618 (0.01)	0.73 (0.07)	0.382 (0.01)	2.55 (0.04)	1.43 (0.06)	8	0.09	0.62	0.07
N/glyc	239	0.602 (0.05)	1.76 (0.2)	0.398 (0.05)	4.98 (0.3)	3.03 (0.05)	4	0.19	1.02	0.12
NADP	298	0.539 (0.07)	0.67 (0.1)	0.461 (0.07)	2.58 (0.1)	1.55 (0.07)	5	0.10	0.50	0.07
FAD	298	1.0	2.40 (0.04)			2.40 (0.04)	4			
	274	1.0	3.57 (0.05)			3.57 (0.05)	4			

[a] The mean fluorescence lifetime was defined as $\langle\tau\rangle = \sum\alpha_i\tau_i$ [cf. Kulinski et al. (1987)]. Φ_{rel} is calculated with MR in buffer at 298 K as a reference. In the N/glyc and NADP samples the concentrations of NADP$^+$ and MR were 750 and 8 μM, respectively. The values in parentheses are the standard deviation from n independent experiments.

& Walsh, 1983). We have therefore compared the fluorescence decay of the intact, "unclipped", and the modified "clipped", forms without finding any detectable differences. This supports earlier conclusions that the N-terminal segment is not part of the active site (Fox & Walsh, 1983).

There are many conceivable reasons for observing a multiexponential relaxation of the excited state. One reason could be fluorescent impurities in the preparations. Another is possibly that the FAD molecules bind differently to the two subunits of MR. These two possibilities are hardly the explanation since the fluorescence spectra and the relaxation of the excited state are independent of excitation and emission wavelengths.

Fluctuations in the protein structure may cause a distribution of lifetimes. If these fluctuations are much slower than the rate of fluorescence, a multiexponential decay is expected, whereas rapid fluctuations should result in a monoexponential decay (Szabo, 1984). It should be noticed that the fluorescence decay could very well be fitted to a sum of two exponential functions even if the true relaxation is multiexponential or more complex.

The mean fluorescence lifetime, $\langle\tau\rangle$, of the native protein and the lifetime of free FAD are similar and both increase with decreasing temperature. In free FAD the adenine moiety is known to quench the fluorescence of the isoalloxazine ring statically and dynamically (Weber, 1950; Spencer & Weber, 1972). In contrast to what is found for free FAD, we find that for FAD in MR the quantum yield Φ and $\langle\tau\rangle/\tau_0$ are the same within the experimental precision. Both Φ and $\langle\tau\rangle$ decrease with increasing temperature. The isoalloxazine chromophore per se does not show such a dependence, i.e., the lifetime of FMN changes only slightly with temperature (Visser, 1984). This implies that FAD in MR is only quenched dynamically. $\langle\tau\rangle$ is about the same in water and in a glycerol–water mixture, where the difference in viscosity is 10-fold. This is compatible with one or more quenchers localized close to the isoalloxazine ring and, furthermore, that these are not exposed to the solvent.

The in vitro activation of MR involves a reduction of a disulfide bond between Cys$_{558}$ and Cys$_{559}$ (Miller et al., 1989) and is associated with a decrease of the quantum yield and the mean lifetime by 38% and 37%, respectively (cf. Table I). Thereby, Φ and $\langle\tau\rangle$ are reduced to the same extent, implying a dynamic quenching. The most straightforward explanation is that the reduced disulfide is in the active site and in the vicinity of the isoalloxazine ring although not in direct contact with it (Miller et al., 1989). Another explanation that cannot be excluded is that the in vitro activation may change the internal mobility so that some other amino acid or amino acids are responsible for the quenching. In that case the C-terminal disulfide does not need to be near the active site.

Compared to many other flavoproteins MR has an unusually high quantum yield. The quantum yields of lipoamide dehydrogenase, GR, and NADPH-cytochrome P-450 reductase are about 0.1, 0.01, and 0.001, respectively (Visser et al., 1980; de Kok & Visser, 1987; Bastiaens et al., 1989). It has been proposed that the fluorescence of GR is quenched by the disulfide bridge between Cys$_{58}$ and Cys$_{63}$ (de Kok & Visser, 1987), which is located close to the isoalloxazine ring (Karplus & Schulz, 1987). It is known that the isoalloxazine ring may be quenched by sulfur-containing amino acids (Penzer & Radda, 1967) and thioethers (Falck & McCormick, 1976). With this explanation the distance between the disulfide bridge Cys$_{135}$–Cys$_{140}$ and the isoalloxazine ring in MR should be larger than the corresponding distance in GR. This is probably not the case since the relative intensities of the charge-transfer bands are similar in GR and MR.

The different quantum yields of MR and GR could also arise from differences in the amino acid sequences in the FAD binding regions. There are extensive homologies in these domains (Rice et al., 1984), but there is also an interesting difference. The Tyr$_{197}$ in human GR is located in the NADPH-binding pocket close to the isoalloxazine ring (Pai & Schulz, 1983). This residue is not conserved in MR where Val$_{279}$ is located in the corresponding position. Tyrosine is a well-known quencher of flavins (Penzer & Radda, 1967). Berry et al. (1989) have replaced Tyr$_{177}$ in the homologous GR of *E. coli* with Phe, Ser, and Gly. All of the mutants exhibited about 25 times higher quantum yield as compared to the wild-type enzyme of *E. coli*. Hence, a plausible explanation of the high quantum yield of MR is the absence of a quenching amino acid in position 279. It is therefore not surprising that also lipoamide dehydrogenase, which has isoleucine in the corresponding position, has a quantum yield close to that of MR.

Time-Resolved Anisotropy. The anisotropy $r(t)$ is an angular correlation function. In the absence of coupling between the reorientational motions and the relaxation of the excited state of a fluorophore, $r(t)$ describes the mutual orientation of the absorption and emission transition moments at the times of excitation and emission (Szabo, 1984). For the FAD molecules in MR, three processes can contribute to the decay of $r(t)$: namely, the overall tumbling of the protein, the local motions of the isoalloxazine rings, and electronic energy transfer between the flavins in the two subunits.

The decay parameters of the fluorescence anisotropy for FAD in MR are summarized in Table II. Most of the $r(t)$ data could be fitted to a single-exponential function.

The rotational correlation time ϕ of a spherical molecule

Table II: Fluorescence Anisotropy Parameters Obtained for Different Samples of Mercuric Reductase[a]

MR	T (K)	β_1	ϕ_1 (ns)	β_2	ϕ_2 (ns)	n	$r(0)$	r_s	χ^2	$\eta T_0/\eta_0 T$
native	298	0.362 (0.001)	39 (2)			6	0.362	0.341	1.03	1
	274	0.367 (0.001)	48 (2)			4	0.367	0.345	1.10	2.11
glyc	298	0.365 (0.001)	54 (3)			6	0.365	0.350	0.90	9.9
	274	0.364 (0.001)	59 (2)			4	0.364	0.346	1.44	22.7
	274	0.141 (0.002)	16.0 (1)	0.229 (0.001)	∞	4	0.370	0.346	1.23	22.7
	239	0.369 (0.001)	68 (2)			4	0.369	0.346	1.76	420
	239	0.124 (0.005)	14.7 (1)	0.250 (0.006)	∞	4	0.374	0.346	1.28	420
N/glyc	239	0.369 (0.001)	102 (5)			4	0.369	0.352	1.15	420
	239	0.081 (0.006)	14.5 (2)	0.293 (0.008)	∞	4	0.374	0.352	1.02	420
NADP	298	0.364 (0.001)	52 (6)			5	0.364	0.350	1.20	1

[a] $r(0)$ and r_s are the initial and steady-state anisotropies, ϕ_1 and ϕ_2 are rotational correlation times. Typical values of χ^2 are given. A single fit was considered as acceptable if the statistical parameter χ^2 was between 0.8 and 1.3 [cf. O'Connor and Phillips (1984, p 181)]. The values in parentheses are the standard deviation from n independent experiments. η/η_0 is the relative viscosity and T_0 = 298 K.

in a solvent can be estimated from the Stokes–Einstein equation:

$$\phi = \eta V/kT \tag{11}$$

V is the molecular volume, η the solvent viscosity, k the Boltzmann constant, and T the absolute temperature. For rigid nonspherical molecules, there are at the most five rotational correlation times possible (Ehrenberg & Rigler, 1972). It is not normally possible to resolve them experimentally because they are often of the same magnitude. Another difficulty is connected with the statistical nature of the measuring process. Most often the data can be described by a single effective correlation time. It has been found that the observed correlation times for proteins are a factor of 1.4–2.4 longer than correlation times calculated according to eq 11 (Bucci & Steiner, 1988). This discrepancy can be ascribed to hydration of the protein and a shape factor, accounting for the deviations from spherical symmetry of the protein. For a molecular weight of 100000–118000 (corresponding to the clipped and unclipped forms of MR) and a specific volume of 0.73 g cm^{-3}, the estimated rotational correlation time is 50–100 ns in water at 298 K. From experiments we obtain a correlation time of 39 ns, which is significantly lower than the predicted ϕ.

According to eq 11 ϕ is directly proportional to η/T. We find from $r(t)$ of MR in water that the correlation time increases from 39 to 48 ns as the temperature decreases from 298 to 274 K, while η/T increases by a factor of 2. The discrepancy between the changes of the experimental correlation times and η/T in the glycerol–water system is even worse, as can be seen in Table II. Thus, other depolarization processes contributing to the measured correlation times are present in MR.

The rotational motions of a molecule, like the isoalloxazine ring, occur typically on a timescale of 1 ns or faster. Evidence for fast unresolved decay processes of $r(t)$ can be obtained from a comparison of $r(0)$ and the limiting fluorescence anisotropy, r_0. The value of r_0 corresponds to the anisotropy of noninteracting fluorophores whose rotational motions are negligible, and r_0 appears to be an inherent property of the chromophore (Johansson, 1990). We found that $r_0 = 0.376 \pm 0.005$ for FAD and the initial anisotropy $r(0) = 0.362$ at 298 K. The difference from 0.376 can be ascribed to fast unresolved motions of the isoalloxazine ring. The difference between $r(0)$ and r_0 decreases at lower temperatures and appears to be absent at 239 K. Hence, the small difference between $r(0)$ and r_0 and the very slow decay of $r(t)$ show that the rotational motions of the isoalloxazine rings in MR are strongly restricted but that rapid vibrational motions of small amplitude probably occur at room temperature. This agrees very well with what was suggested for the flavin moiety in GR based on X-ray data (Thieme et al., 1981; Karplus & Schulz, 1987). They found that especially the flavin and also the entire active center were particularly well ordered with temperature factors below 8.7 Å^2 and aroung 10 Å^2, respectively. In contrast, de Kok and Visser (1987) interpreted their fluorescence depolarisation data on GR as if the flavins were very flexible.

For establishing the presence of energy transfer between the FAD molecules in MR, we cooled the glycerol solution to 239 K. The viscosity was then high enough to ensure that the enzyme was immobile on the fluorescence timescale, while the solution was still in the liquid state (Segur, 1953). We found that a monoexponential fit to $r(t)$ was statistically unacceptable, while an exponential function plus a constant term was statistically acceptable, as can be seen in Table II. This was also what was expected from the theory of energy transfer between two identical chromophores in a dimeric system (Tanaka & Mataga, 1979). The anisotropy decay for rigidly attached fluorophores in the presence of energy transfer is given by

$$r(t) = (r_0 - r_\infty)\exp(-2k_{\mathrm{DA}}t) + r_\infty \tag{12}$$

where

$$r_\infty = \frac{1}{2}\left[r_0 + \frac{(3\cos^2\theta - 1)}{5}\right] \tag{13}$$

and θ is the angle between the emission transition dipole in the donor and the absorption transition dipole of the acceptor. By fitting the $r(t)$ data to eq 12 we find a correlation time of energy transfer ($=1/2k_{\mathrm{DA}}$) of 14.7 ns and $r_\infty = 0.250 \pm 0.008$. The value of r_∞ corresponds to $\theta = 43° \pm 2°$ or $\theta = 137° \pm 2°$.

Before we discuss these results in detail it would be informative to present the relevant geometrical data of FAD in GR. The absorption transition dipole of the first electronic transition in the isoalloxazine ring coincides approximately with a straight line connecting the C-4 and C-9 atoms (Johansson et al., 1979). From the X-ray data of GR a distance of 38.6 Å was calculated between the center of the two isoalloxazine rings. The angle between their absorption transition dipoles, θ, is 160°. The angles between the absorption transition dipoles and a straight line passing through the centers of the two isoalloxazine rings, θ_r, are the same because of the C_2 symmetry of GR. The X-ray data yield $\theta_r = 14°$. From θ and θ_r we found that $\kappa^2 = 3.6$ for GR. The absorption and emission transition dipoles were considered to be parallel, and their direction was assumed to be unperturbed by the small, but significant, deviations from the standard flavin geometry, which the flavins bound to GR show (Karplus & Schulz, 1987). By assuming that the overlap integral and the radiative lifetime of GR are equal to those of MR, the correlation time

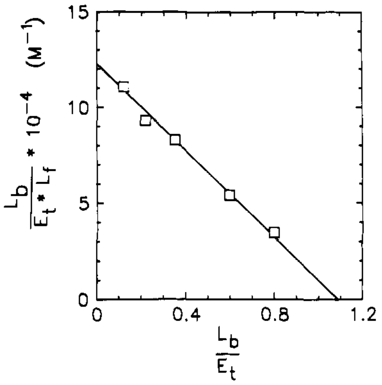

FIGURE 2: Scatchard plot showing the binding of NADP$^+$ to oxidized MR. E_t, L_b, and L_f denote the concentrations of mercuric reductase, bound NADP$^+$, and free NADP$^+$, respectively.

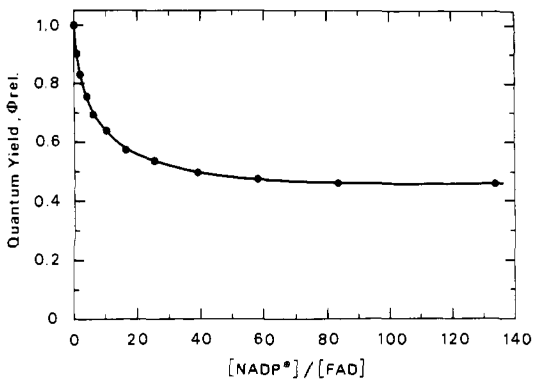

FIGURE 3: Relative fluorescence quantum yield of FAD in mercuric reductase as a function of [NADP$^+$]/[FAD] at 298 K. A 5.22 mM NADP$^+$ solution was successively added to an originally 7.05 μM solution of MR.

of energy transfer ($=1/2k_{DA}$) becomes 5.6 ns. The $r(t)$ obtained when the protein molecules rotate as spherical particles is expected to be biexponential. One of the correlation times corresponds to the rotational motion, while the second one is determined by the rates of rotation and energy transfer. Since both correlation times are long compared to $\langle\tau\rangle$ (=0.11 ns) in GR (de Kok & Visser, 1987), it is in practise hard to separate them.

For MR we obtained two alternatives for θ, namely, 43 ± 2° and 137 ± 2°. If MR also possesses C_2 symmetry the first and second alternatives constrain κ^2 within 0.54–1.28 and 0–3.48, respectively. Provided that the distances between the isoalloxazine rings are within 38.6 ± 1 Å, the only unknown in eq 6 is κ^2. We then find that $\kappa^2 = 1.4 \pm 0.2$. For $\theta = 43°$ and $\kappa^2 = 1.2$ (R = 37.6 Å) it follows tht $\theta_r = 70°$. On the other hand for $\theta = 137°$ and $\kappa^2 = 1.4 \pm 0.2$, one obtains $\theta_r = 37° \pm 2°$. Thus, it is not possible to definitely exclude that $\theta = 43 \pm 2°$, but the alternative of 137 ± 2° seems more conformable to GR and hence also more probable. Our data strongly suggests that the flavins are tilted in a slightly different way in MR than in GR, although the above-mentioned assumptions should be borne in mind.

Titration with NADP$^+$. The association between MR and NADP$^+$ was measured by means of equilibrium dialysis and a combined titration–fluorescence study. A Scatchard plot of data obtained from equilibrium dialysis (Figure 2) yielded an association stochiometry of one NADP$^+$ per subunit and a dissociation constant of about 9 μM. Previously, a dissociation constant of about 10 μM was also obtained from absorption experiments (Sahlman et al., 1984).

The ratio between the quantum yield of MR as a function of [NADP$^+$]/[FAD] and the quantum yield of unliganded MR at that temperature was measured. A typical fluorescence quenching curve is shown in Figure 3. The relative quantum yield decreases monotonically and reaches a plateau value. The level of this value is temperature dependent and varies between 0.4 and 0.65 in the temperature range of 274–313 K. At 298 K, $\langle\tau\rangle$ is reduced to about 70% of the value for the native protein while Φ is reduced to about 50%. Accordingly the quenching must be both dynamic and static. Exchanging the solvent for the glycerol/buffer mixture and cooling to 239 K do not alter the situation: $\langle\tau\rangle$ and Φ are reduced to the same extent as mentioned above. This suggests that the quencher, very likely the nicotinamide moiety of NADP$^+$, is not directly exposed to the solvent and that it has some freedom of movement in the protein matrix. This interpretation is in part supported by the crystallographic studies of NADP$^+$ binding to GR (Pai et al., 1988). They found only 15% of the expected electron density of the nicotinamide moiety in the nicotinamide pocket. The location of the moiety when not bound to the pocket could not be resolved.

Measurements on NADP$^+$-liganded MR in the glycerol/buffer solution at 239 K show that the anisotropy decay could be fitted to a monoexponential decay with a correlation time of 102 ns. A better fit was obtained with an exponential function plus a constant term. The correlation time was then 14.5 ns, which is the same as obtained for unliganded MR in the glycerol/buffer solution. The constant term was 0.293 as compared to 0.250 for unliganded MR. These observations could be explained by the following model for the binding of NADP$^+$ to MR: when adding NADP$^+$ in great excess to MR, all subunits associate with one NADP$^+$ molecule each. At every moment some of the subunits bind the nicotinamide moiety very close to the flavin, which in turn is quenched statically. At the same moment other subunits do not bind the nicotinamide moiety close enough to the flavin to quench it statically but still close enough for it to possibly quench the flavin dynamically. The enzyme solution will then contain a mixture of dimers where both flavins are quenched statically, dimers where only one of the flavins is quenched statically, and dimers where none of the flavins is quenched statically. For the dimers where none of the flavins is quenched statically, the fluoresence anisotropy will decay in the same way as for the enzyme when unassociated with NADP$^+$; i.e., the anisotropy will decay as a monoexponential function plus a constant term if all motions are absent. For the dimers where only one flavin is quenched statically, the anisotropy will decay only as a consequence of the motions of the protein. Energy transfer to statically quenched chromophores does not contribute to the anisotropy decay. It only shows up as a shortening of the lifetime. In the absence of motions, the anisotropy will be constant and equal to r_0 for this fraction of dimers. The dimers where both flavins are quenched statically do not fluoresce at all.

Taken together, for a rigid solution of such a mixture the anisotropy decay is an exponential function plus a constant term. The correlation time is determined by the rate of energy transfer as before, but the constant term is now dependent on the size of the fraction of dimers where only one flavin is quenched statically. This model also gives a rational explanation for why the (effective) correlation time increases from 39 to 52 ns when adding NADP$^+$ to MR in the buffer solution at 298 K.

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REFERENCES

Bastiaens, P. I. H., Bonants, P. J. M., Müller, F., & Visser, A. J. W. G. (1989) *Biochemistry 28*, 8416–8425.

Bernofsky, C. (1980) *Methods Enzymol. 66*, 23–39.

Berry, A., Scrutton, N. S., & Perham, R. N. (1989) *Biochemistry 28*, 1264–1269.

Birks, J. B. (1970) *Photophysics of Aromatic Molecules*, Wiley-Interscience, London.

Brown, N. L., Ford, S. J., Pridmore, R. D., & Fritzinger, D. C. (1983) *Biochemistry 22*, 4089–4095.

Bucci, E., & Steiner, R. F. (1988) *Biophys. Chem. 30*, 199–224.

Carothers, D. J., Pons, G., & Patel, M. S. (1989) *Arch. Biochem. Biophys. 268*, 409–425.

Dale, R. E., Eisinger, J., & Blumberg, W. E. (1979) *Biophys. J. 26*, 161–194.

de Kok, A., & Visser, A. J. W. G. (1987) *FEBS Lett. 218*, 135–138.

Drexhage, K. H. (1977) in *Laser Dyes* (Schäfer, F. P., Ed.) 2nd ed., Springer-Verlag, Berlin, Heidelberg, New York.

Ehrenberg, M., & Rigler, R. (1972) *Chem. Phys. Lett. 14*, 539–544.

Falck, M. C., & McCormick, D. B. (1976) *Biochemistry 15*, 646–653.

Förster, T. (1948) *Ann. Phys. 2*, 55–75.

Förster, T. (1965) in *Modern Quantum Chemistry*, Istanbul Lectures (Sinanoğlu, O., Ed.) Part 3, pp 93–137, Academic Press, New York.

Fox, B., & Walsh, C. T. (1982) *J. Biol. Chem. 257*, 2498–2503.

Fox, B., & Walsh, C. T. (1983) *Biochemistry 22*, 4082–4088.

Johansson, L. B.-Å. (1990) *J. Chem. Soc., Faraday Trans. 86*, 2103–2107.

Johansson, L. B.-Å., Davidsson, Å., Lindblom, G., & Naqvi, K. R. (1979) *Biochemistry 18*, 4249–4253.

Karplus, P. A., & Schulz, G. E. (1987) *J. Mol. Biol. 195*, 701–729.

Kubin, R. F., & Fletcher, A. N. (1982) *J. Luminescence 27*, 455–462.

Kulinski, T., Visser, A. J. W. G., O'Kane, D. J., & Lee, J. (1987) *Biochemistry 26*, 540–549.

Miller, S. M., Moore, M. J., Massey, V., Williams, C. H., Jr., Distefano, M. D., Ballou, D. P., & Walsh, C. T. (1989) *Biochemistry 28*, 1194–1205.

O'Connor, D. V., & Phillips, D. (1984) *Time-correlated Single Photon Counting*, Academic Press, Orlando, FL.

Pai, E. F., & Schulz, G. E. (1983) *J. Biol. Chem. 258*, 1752–1757.

Pai, E. F., Karplus, P. A., & Schulz, G. E. (1988) *Biochemistry 27*, 4465–4474.

Penzer, G. R., & Radda, G. K. (1967) *Q. Rev. Chem. Soc. 21*, 43–65.

Rice, D. W., Schulz, G. E., & Guest, J. R. (1984) *J. Mol. Biol. 174*, 483–496.

Sahlman, L., & Lindskog, S. (1983) *Biochem. Biophys. Res. Commun. 117*, 231–237.

Sahlman, L., Lambeir, A.-M., Lindskog, S., & Dunford, H. B. (1984) *J. Biol. Chem. 259*, 12403–12408.

Sandström, A., & Lindskog, S. (1988) *Eur. J. Biochem. 173*, 411–415.

Schierbeek, A. J., Swarte, M. B. A., Dijkstra, B. W., Vriend, G., Read, R. J., Hol, W. G. J., Drenth, J., & Betzel, C. (1989) *J. Mol. Biol. 206*, 365–379.

Segur, J. B. (1953) in *Glycerol* (Miner, C. S., & Dalton, N. N., Eds.) pp 238–334, American Chemical Society, Washington, DC.

Shames, S. L., Fairlamb, A. H., Cerami, A., & Walsh, C. T. (1986) *Biochemistry 25*, 3519–3526.

Spencer, R. D. & Weber, G. (1972) in *Structure and Function of Oxidation Reduction Enzymes* (Åkesson, Å., & Ehrenberg, A., Eds.) pp 393–399, Pergamon Press, Oxford.

Szabo, A. (1984) *J. Chem. Phys. 81*, 150–167.

Tanaka, F., & Mataga, N. (1979) *Photochem. Photobiol. 29*, 1091–1097.

Thieme, R., Pai, E. F., Schirmer, R. H., & Schulz, G. E. (1981) *J. Mol. Biol. 152*, 763–782.

Valeur, B., & Weber, G. (1977) *Photochem. Photobiol. 25*, 441–450.

Wang, T. P., Kaplan, N. O., & Stolzenbach, F. E. (1954) *J. Biol. Chem. 211*, 465–472.

Weber, G. (1950) *Biochem. J. 47*, 114–121.

Westphal, A. H., & de Kok, A. (1988) *Eur. J. Biochem. 268*, 299–305.

Williams, C. H., Jr. (1976) *Enzymes (3rd Ed.) 13*, 89–173.

Visser, A. J. W. G. (1984) *Photochem. Photobiol. 40*, 703–706.

Visser, A. J. W. G., Grande, H. J., & Veeger, C. (1980) *Biophys. Chem. 12*, 35–49.