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Time-Resolved Fluorescence Spectroscopy of NADPH-Cytochrome P-450 Reductase: Demonstration of Energy Transfer between the Two Prosthetic Groups†

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ABSTRACT: Fluorescence as well as fluorescence anisotropy decay parameters have been obtained from NADPH-cytochrome P-450 reductase by time-resolved fluorescence spectroscopy. The two flavins in the enzyme, FMN and FAD, are slightly fluorescent and exhibit heterogeneous fluorescence lifetimes, as observed with other flavoproteins. The time-dependent anisotropy is also multiexponential and is wavelength-dependent. The anisotropy decay is biexponential with two correlation times when the enzyme is excited at the red edge of the first absorption band (514 nm). When the enzyme is excited in the light absorption maximum (458 nm), an additional shorter correlation time is found, which contains information about the rate of energy transfer between the two flavins present in the enzyme. FMN-depleted NADPH-cytochrome P-450 reductase shows also only two correlation times, as does the enzyme in the "air-stable" semiquinone state when excited at 458 nm. Wavelength-dependent steady-state anisotropy measurements of native and FMN-depleted protein show that the former exhibits lower values than the latter in the region of the first absorption band, but when the red edge of the absorption band is reached, the anisotropy becomes equal in both preparations. A similar situation is encountered in model compounds, monomeric and dimeric flavins, immobilized in poly(methyl methacrylate). Both in the models and in the flavoprotein this can be attributed to failure of energy transfer at the red edge of the absorption band. From the results we were able to derive both geometric parameters and dynamic properties of both flavins in the NADPH-cytochrome P-450 reductase. These data indicate that energy transfer occurs between the prosthetic groups in NADPH-cytochrome P-450 reductase and that the distance between the two flavins is about 2 nm. The results are briefly discussed with regard to the biochemical significance of the data.

NADPH-cytochrome P-450 reductase (EC 1.6.2.4) from mammalian liver is an unusual flavoprotein that contains two prosthetic groups, i.e., riboflavin 5′-phosphate (FMN)[1] and flavin adenine dinucleotide (FAD) (Iyanagi & Mason, 1973; Masters et al., 1975; Vermillion & Coon, 1974). The native membrane-bound protein possesses a molecular mass of 78 kDa. By proteolytic treatment of the protein a 68-kDa fragment of the enzyme can be obtained in a pure form. This reductase is no longer able to transfer electrons from NADPH to cytochrome P-450, but it still catalyzes electron transfer to the artificial acceptor cytochrome *c*. Obviously, the hydrophobic part of native reductase, which is lost upon proteolysis, is responsible for the proper interaction with cyto-

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[1] Abbreviations: FMN, oxidized riboflavin 5′-phosphate; FMNH, neutral one-electron-reduced riboflavin 5′-phosphate; FAD, oxidized flavin adenine dinucleotide; FWHM, full width at half-maximum; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; PMSF, phenylmethanesulfonyl fluoride; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; MLF, methyllumiflavin; TCSPC, time-correlated single-photon counting.

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chrome P-450. An FMN-depleted form and a rather stable semiquinone, referred to in the literature as the "air-stable" radical, can be formed from both enzyme preparations. This suggests that the basic chemical and structural properties of the reductase are not affected when treated with proteases.

Although NADPH–cytochrome P-450 reductase plays an important role in the metabolism of xenobiotics, little structural information is as yet available. An outstanding problem in NADPH–cytochrome P-450 reductase is that we still know relatively little about the spatial relationship between the two flavins. There are only a few physical techniques that might shed light on this problem, and only a few physical studies have been carried out with NADPH–cytochrome P-450 reductase, i.e., a resonance Raman and a ^{31}P NMR study. The resonance Raman study (Sugiyama et al., 1985) revealed that the interactions of the flavin(s) with the protein in the native and FMN-depleted form are identical. These interactions are altered in the semiquinone state of the protein. The ^{31}P NMR study (Otvos et al., 1986) proved unequivocally that the unpaired electron in the "air-stable" semiquinone form of the reductase resides on FMN in agreement with suggestions deduced from light absorption studies (Vermillion & Coon, 1978a; Iyanagi et al., 1974, 1978). A technique more suitable to obtain the desired spatial information is time-resolved fluorescence. Such a study was carried out by Blumberg et al. (1982). It was concluded that energy transfer possibly occurs between the two flavins in the reductase. However, the experimental data obtained at that time did not allow firm conclusions to be drawn from the data. The time-correlated single-photon counting (TCSPC) fluorescence technique with a laser as excitation source has undergone rapid development since that time and reached a high degree of sophistication. Owing to the high dynamic range of intensity and good time resolution of such instruments, very weakly fluorescent compounds can be investigated and the relevant dynamic information can be extracted from the experimental results by well-established computational methods [see, for instance, Cundall and Dale (1983), Brand et al. (1985), Lakowicz (1988), and Jameson and Reinhart (1989)].

Therefore, we decided to reinvestigate NADPH–cytochrome P-450 reductase by this technique in order to obtain more detailed information about the existence or absence of energy transfer between the two flavins in the enzyme. The results are also of general interest in understanding the mechanism of electron transfer catalyzed by flavoproteins, for example, to answer the question: must there be interflavin orbital contact for electron transfer to occur?

In this paper, the complex time-resolved fluorescence properties of the protein are reported. Comparing the anisotropy decay of the native protein excited at the first absorption maximum and at the red edge of the absorption band, it was concluded that energy transfer between the two flavins does indeed take place. The results are interpreted with the aid of several different models. Preliminary results of this study have been published elsewhere (Bonants et al., 1985, 1987; Bastiaens et al., 1988).

Materials and Methods

Isolation of the Enzyme. NADPH–cytochrome P-450 reductase was isolated from liver microsomes of phenobarbital-treated rats and purified, except for minor modifications, by a published procedure (Yasukochi & Masters, 1976). After solubilization of the enzyme, it was purified by DEAE ion-exchange chromatography followed by 2′,5′-ADP–Sepharose affinity chromatography. As judged by SDS–PAGE according to Laemmli (1970), the reductase was homogeneous. The molecular mass of the enzyme was 78 kDa, as reported before (Yasukochi & Masters, 1976; Vermillion & Coon, 1978b; Coon et al., 1977; Guengerich, 1978). Activity was measured at 25 °C in 0.3 M potassium phosphate, pH 7.7, by monitoring the reduction of cytochrome *c* at 550 nm ($\Delta\epsilon$ = 21.0 mM^{-1} cm^{-1}).

Preparation of FMN-Depleted Enzyme. A 1-mL aliquot of a 30 μM stock solution of NADPH-cytochrome P-450 reductase was diluted into 400 mL of "apo buffer" (100 mM Tris-HCl, containing 20% glycerol, 2 M KBr, 0.1 mM EDTA, 1.0 mM DTT, 0.1 mM PMSF, pH 8.4) and concentrated in an Amicon ultrafiltration apparatus (400 mL; YM-30 filter) at 4 °C. After concentration of the sample it was again diluted with apo buffer and concentrated. This procedure was repeated several times until FMN was completely removed. After the final concentration step, KBr was removed from the FMN-depleted enzyme by gel filtration (Bio-Gel P-6DG, 25 mM potassium phosphate, containing 20% glycerol, 0.1% lubrol, 0.1 mM DTT, 0.1 mM EDTA, pH 7.7). The content of residual FMN in the sample was analyzed according to the procedure described by Wassink and Mayhew (1975). The activity of the FMN-depleted reductase was measured with and without incubation with FMN.

Instrumentation. The TCSPC apparatus, consisting of an argon ion laser and associated optics and electronics, has been extensively described earlier (Visser & Van Hoek, 1979; Van Hoek & Visser, 1985; Visser et al., 1985). Excitation wavelengths used were 458 or 514 nm; the emission wavelengths were selected with Balzers interference filters transmitting at 531 or 550 nm, with half-bandwidths of 10 nm. Measurements were carried out at 10 °C.

Steady-State Fluorescence. Steady-state fluorescence was measured on an instrument described by Jameson et al. (1978). The excitation bandwidth was 3.3 nm. Emission wavelengths were selected by Corning 3-67 and 3-69 sharp-cut and 4-94 broad-band filters. Measurements were performed at 10 °C, and 15 measurements were averaged. Fluorescence quantum yields were calculated with FMN as a reference [quantum yield = 0.26 (Weber & Teale, 1957)].

Sample Preparations. The samples were measured in 25 mM potassium phosphate containing 20% glycerol, 0.1% lubrol, 0.1 mM EDTA, and 0.1 mM DTT, pH 7.7. For recombination of the FMN-depleted enzyme, the protein was incubated with an excess of FMN at 4 °C for 1 h. Excess FMN was then removed by gel filtration. The semiquinone (one-electron-reduced state) of the native reductase was made by addition of small amounts of a dithionite solution under anaerobic conditions. The reduction was followed spectrophotometrically to ensure complete formation of the semiquinone. The solution was made anaerobic with a vacuum argon line and was flushed with argon. This cycle was repeated several times.

The model compounds N^3-methyllumiflavin and N^{10},$N^{10\prime}$-trimethylene- and N^{10},$N^{10\prime}$-hexamethylenebis-(3,7,8-trimethylisoalloxazine) were prepared according to published procedures (Leonard & Lambert, 1969). The incorporation of these compounds into poly(methyl methacrylate) has been described previously (Visser et al., 1983). As a pulse mimic reference compound, erythrosin B (Eastman Kodak) in water was used. Methanol was fluorescent grade from Merck.

Viscosity. The viscosity of the buffer was measured at 10 °C on an Ostwald viscometer.

Data Analysis. Data analysis for the fluorescence and anisotropy decays was performed with an iterative deconvo-

lution method (Vos et al., 1987) using erythrosin B in water as reference compound.

The decays of fluorescence $I(t)$ and anisotropy $r(t)$ were fitted with multiexponential functions until a minimum reduced χ^2 near 1 was obtained:

$$I(t) = \sum_i \alpha_i e^{-t/\tau_i} \quad (1)$$

$$r(t) = \sum_i \beta_i e^{-t/\phi_i} \quad (2)$$

Energy Transfer. Electronic excitation energy can be transferred between appropriate pairs of chromophores. A general requirement for excitation transfer is that the donor fluorescence spectrum overlaps the acceptor absorption spectrum. For dipole–dipole interaction the specific rate of excitation transfer k_{DA} between an excited donor molecule (D) and an acceptor molecule (A) separated by a distance R (in angstroms) is given by the Förster equations (Förster, 1948):

$$k_{DA} = 8.784 \times 10^{23}\, J(\lambda)\, \kappa^2 Q_f \tau_{D_0}^{-1} n^{-4} R^{-6}\ (s^{-1}) \quad (3)$$

and

$$k_{DA} = (1/\tau_{D_0})(R_0/R)^6 \quad (4)$$

where n is the refractive index of the medium between D and A, Q_f is the donor fluorescence quantum yield in the absence of acceptor, τ_{D_0} is the fluorescence lifetime of the donor in the absence of acceptor, R_0 is the critical separation between D and A at which half of the excitation energy is transferred, and $J(\lambda)$ is the spectral overlap integral defined by

$$J(\lambda) = \frac{\int_0^\infty F_D(\lambda)\, \epsilon_A(\lambda)\, \lambda^4\, d\lambda}{\int_0^\infty F_D(\lambda)\, d\lambda}\ (cm^3\ M^{-1}) \quad (5)$$

where $F_D(\lambda)$ is the fluorescence intensity of the donor at wavelength λ (in cm) and $\epsilon_A(\lambda)$ is the molar extinction coefficient (in $M^{-1}\ cm^{-1}$) of the acceptor. κ^2 is an orientation factor that depends on the relative orientations of the donor and acceptor molecules and is defined by

$$\kappa^2 = [\hat{\mathbf{a}}\cdot\hat{\mathbf{d}} - 3(\hat{\mathbf{a}}\cdot\hat{\mathbf{r}})(\hat{\mathbf{d}}\cdot\hat{\mathbf{r}})]^2 \quad (6)$$

where $\hat{\mathbf{a}}$ is a unit vector along the absorption transition moment of A, $\hat{\mathbf{d}}$ a unit vector along the emission transition moment of D, and $\hat{\mathbf{r}}$ a unit vector along the direction between the absorption and emission transition moments of A and D.

In the case of heterotransfer (from oxidized flavin to semiquinone), the k_{DA} is determined experimentally by measuring the lifetime of the donor in the absence (τ_{D_0}) and the presence (τ_D) of the acceptor. The relationship can be written (Steinberg, 1971) as

$$k_{DA} = 1/\tau_D - 1/\tau_{D_0} \quad (7)$$

While, in principle, R could be computed from eq 3, it has become customary to calculate the so-called Förster critical distance, R_0, the distance where the transfer rate k_{DA} is equal to the decay rate of the donor ($\tau_{D_0}^{-1}$) in the absence of acceptor:

$$R_0^6 = (8.784 \times 10^{-25}) Q_f \kappa^2 n^{-4}\, J(\lambda)\ (cm^6) \quad (8)$$

Interpretation of Time-Resolved Fluorescence Anisotropy. In the enzyme studied, there are three possible contributions to the fluorescence depolarization: (i) the overall motion of the protein , (ii) the motional freedom of the flavins with respect to the protein as a whole, and (iii) energy transfer between the two flavins.

In this section an approximate expression will be derived for the time-dependent anisotropy of dimeric systems in which energy transfer between like fluorophores (homotransfer) takes place, and which exhibit restricted rotational motion. By a dimeric system we thus understand a unit of two weakly interacting transition dipoles belonging to identical chromophores bound to a macromolecule or covalently bound by a spacer.

When the reorientation of the monomers takes place on a time scale comparable to the reciprocal rate of energy transfer, the exact mathematical expression for the time-resolved anisotropy becomes extremely complex. In that case, the orientation factor, κ^2, and consequently the rate of transfer, k_T, will be time-dependent. For high orientational order of the interacting moieties, the variation of κ^2 with time will be small and the orientation factor can be approximated by a constant. It can be deduced that, for δ excitation, the anisotropy decay for a dimeric system where the two monomers interact by Förster-type energy transfer is given (Szabo, 1984) by

$$r(t) = 2\sum_{i,j=1}^{2} p[i(t)|j(0)]\langle\langle P_2(u_a^i \cdot u_{e_t}^j)\rangle\rangle / 5\sum_{i,j=1}^{2} p[i(t)|j(0)] \quad (9)$$

where $p[i(t)|j(0)]$ is the conditional probability that the system is in electronic state i at time t given it was in state j at time $t = 0$. For a two-state problem analytical expressions for the conditional probabilities of the electronic states have been given, among others, by Förster (1948) and, recently, by Szabo (1984):

$$p[1(t)|1(0)] = p[2(t)|2(0)] = \tfrac{1}{2}[\exp(-k_F t) + \exp[-(k_F + 2k_T)t]] \quad (10)$$

$$p[1(t)|2(0)] = p[2(t)|1(0)] = \tfrac{1}{2}[\exp(-k_F t) - \exp[-(k_F + 2k_T)t]] \quad (11)$$

where k_F is the fluorescence rate in the absence of Förster energy transfer and k_T is the rate of energy transfer. For $i = j$, $\langle\langle P_2(u_a^i \cdot u_{e_t}^i)\rangle\rangle$ is the orientational correlation function of the monomers defined by

$$\langle\langle P_2(\mathbf{u}_a^i \cdot \mathbf{u}_{e_t}^i)\rangle\rangle = \int_\Omega \int_{\Omega_0} d\Omega\, d\Omega_0\, P_2[\mathbf{u}_a^i(\Omega_0)\cdot\mathbf{u}_e^i(\Omega)]\, p[\Omega(t)|\Omega_0(0)]\, p_{eq}(\Omega_0) \quad (12)$$

where $P_2[\mathbf{u}_a^i(\Omega_0)\cdot\mathbf{u}_e^i(\Omega)]$ is the second-order Legendre polynomial of the vector product of absorption transition moment $\mathbf{u}_a^i(\Omega)$, with orientation Ω_0 at time $t = 0$, and emission transition moment $\mathbf{u}_e^i(\Omega)$, with orientation Ω at time t, of molecule i. $p(\Omega(t)|\Omega_0(0)]$ is the conditional probability that the molecule has orientation Ω at time t given it had orientation Ω_0 at time $t = 0$. $p_{eq}(\Omega_0)$ is the equilibrium orientational distribution of the monomers. For $i \neq j$ $\langle\langle P_2(\mathbf{u}_a^i \cdot \mathbf{u}_{e_t}^j)\rangle\rangle$ is the transfer correlation function which can be expanded as a so-called Soleillet product (Soleillet, 1929) in the case that the orientational and electronic states are uncoupled:

$$\langle\langle P_2(\mathbf{u}_a^i \cdot \mathbf{u}_{e_t}^j)\rangle\rangle = \langle\langle P_2(\mathbf{u}_a^i \cdot \mathbf{u}_{e_t}^i)\rangle\rangle P_2(\mathbf{D}_1^x \cdot \mathbf{D}_2^x) \quad (13)$$

where $P_2(\mathbf{D}_1^x \cdot \mathbf{D}_2^x)$ is the second-order Legendre polynomial of the vector product $\mathbf{D}_1^x \cdot \mathbf{D}_2^x$ of the symmetry axes of the potential $U(\Omega)$ in which the probes can reorient.

For a molecule that can reorient in a uniaxial potential one should consider two possibilities. First, the molecule spins around its long symmetry axis, and either its absorption or emission moment coincides with this axis. Second, neither transition moment coincides with the long symmetry axis (which pertains to the flavin molecule), and the molecule does not spin around this axis. In either case, one can use the monoexponential approximation of Van der Meer et al. (1984)

as an analytical expression for the reorientational correlation function of a probe reorientating in a uniaxial potential:

$$\langle\langle P_2(\mathbf{u}_a^i \cdot \mathbf{u}_{e_t}^i)\rangle\rangle = P_2(\cos\delta)[(1 - S_i^2)\exp\{-6D^i_\perp t/(1 - S_i^2)\} + S_i^2] \quad (14)$$

where δ is the angle between the absorption and emission transition moments, S_i is the second-rank order parameter of monomer i, and $D^i_\perp$ is the diffusion coefficient of monomer i. When the tumbling of the macromolecular structure has to be included, eq 14 becomes (Zannoni, 1981)

$$\langle\langle P_2(\mathbf{u}_a^i \cdot \mathbf{u}_{e_t}^i)\rangle\rangle = P_2(\cos\delta) \times [(1 - S_i^2)\exp\{-6D^i_\perp t/(1 - S_i^2)\} + S_i^2]\exp(-t/\phi_p) \quad (15)$$

where it is assumed that this tumbling takes place on a much longer time scale than the restricted reorientational dynamics of the probes, taken to be identical for both monomers. After substitution of eq 10, 11, 13, and 15 into eq 9, we obtain an approximate expression for the time-dependent anisotropy of a dimeric homotransfer system exhibiting restricted reorientational motion of the monomers:

$$r(t) = {}^1\!/_5 P_2(\cos\delta)[\{1 - P_2(\mathbf{D}_1^x \cdot \mathbf{D}_2^x)\}\exp(-2k_T t) + (1 - S^2)\{1 + P_2(\mathbf{D}_1^x \cdot \mathbf{D}_2^x)\}\exp(-t/\phi_{eff}) + S^2\{1 + P_2(\mathbf{D}_1^x \cdot \mathbf{D}_2^x)\}]\exp(-t/\phi_p) \quad (16)$$

where

$$\phi_{eff} = (1 - S^2)/6D_\perp \quad (17)$$

and ϕ_p is the correlation time for macromolecular tumbling. For a spherically symmetric rotor, ϕ_p (in seconds) is given by

$$\phi_p = \eta V/kT \quad (18)$$

where V is the molecular volume of the macromolecular structure (cm^3), η is the viscosity of the solvent (cP), T the absolute temperature (K), and k the Boltzmann constant (J/K).

Equation 16 is complicated by an ambiguity in the roles of energy transfer and rotational depolarization. The observed correlation times might arise exclusively from the rotational freedom of the luminophores and have nothing to do with energy transfer.

One approach to solve this problem would be to alter the system in a chemical or biochemical way such that only monomers are present. In biological systems this is often hard to achieve. In addition, removal of one of the chromophores can alter the physical properties of the other one and therefore make valid comparison difficult. Another, physical, approach is to make use of the failure of homotransfer in dimeric systems composed of identical monomers that frequently takes place upon excitation at the red edge of the absorption band of the first electronic transition. This phenomenon was first observed by Weber and Shinitzky (1970) in dimeric systems dissolved in solvents of high viscosity. It is known that failure of energy transfer occurs upon red-edge excitation when the dipolar relaxation time of the chromophore environment is comparable to or longer than the fluorescence lifetime, i.e., $\tau_r > \tau_F$. Upon red-edge excitation of dimer systems, those molecules are photoselected that have a minimal energy separation between ground and excited states. Because there is no dipolar relaxation on a time scale comparable to the fluorescence lifetime, the excited molecules maintain their energy separation during the lifetime of the excited state. The probability that an acceptor has a comparable or smaller energy separation decreases as one excites the sample at increasingly longer wavelengths at the red edge of the absorption band. When the dipolar relaxation time is shorter than the fluorescent lifetime, so-called up-relaxation takes place after edge excitation. In such cases the molecules will have an energy configuration independent of excitation wavelength, and energy transfer can take place (Demchenko, 1987).

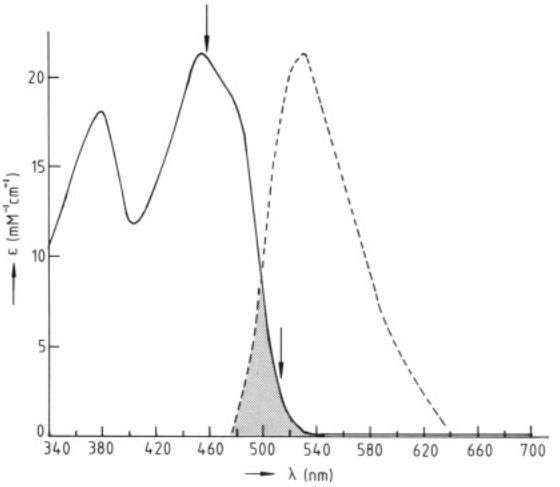

FIGURE 1: Spectral overlap (shaded area) between the absorption (solid line) and emission (dashed line, excitation at 458 nm) spectra of native NADPH–cytochrome P-450 reductase. The arrows indicate the laser excitation wavelengths used in this work.

Upon excitation at the red edge of the absorption band in a system where $\tau_r > \tau_F$ holds, eq 16 becomes

$$r(t) = {}^2\!/_5 P_2(\cos\delta)[(1 - S^2)\exp(-t/\phi_{eff}) + S^2]\exp(-t/\phi_p) \quad (19)$$

In eq 19 the effective relaxation time of restricted fluorophore motion and the rotational correlation time of the protein are the only parameters. We are now able to assign a depolarizing factor to energy transfer, to quantify the restricted fluorophore motion, and finally to calculate an average rate of transfer. With this information one is able to determine an interchromophore distance provided that the overlap integral, excited-state lifetime, and radiative lifetime (or quantum yield) of the system are known.

An important parameter to be determined is the orientational factor κ^2. Dale et al. (1979) developed contour plots to determine boundaries for κ^2 given the axial depolarization factors of both acceptor and donor and the transfer depolarization factor. If the rotational freedom of both monomers in a homotransfer system is identical, the former information can be obtained from the anisotropy of a sample excited at the red edge of the absorption band where eq 19 holds and used to derive the latter from anisotropy determined for excitation in the body of the absorption spectrum. From the preexponential amplitudes the axial orientation factors $\langle d^x\rangle$ and the transfer depolarization factors $\langle d_T^x\rangle$ and d_T^x can be calculated (Dale et al., 1979). In our nomenclature the axial orientation factor $\langle d^x\rangle$ is identical with the order parameter S (eq 14), while the transfer depolarization factor d_T^x is identical with $P_2(\mathbf{D}_1^x \cdot \mathbf{D}_2^x)$ (eq 16) and $\langle d_T^x\rangle$ with the product $S_D d_T^x S_A$.

Results

Spectral Data. In Figure 1 absorption and emission spectra of the native NADPH–cytochrome P-450 reductase are shown in order to illustrate the spectral overlap, indicating that energy transfer is possible between the oxidized FAD and the oxidized FMN and vice versa. The calculated overlap integral (eq 5)

Table I: Fluorescence Lifetime Parameters of Different Samples of NADPH–Cytochrome P-450 Reductase[a]

sample	λ_{ex} (nm)	α_1	τ_1 (ns)	α_2	τ_2 (ns)	α_3	τ_3 (ns)	α_4	τ_4 (ns)	$\langle\tau\rangle^b$ (ns)
native	458	0.616 (0.038)	0.084 (0.010)	0.143 (0.009)	0.56 (0.05)	0.087 (0.004)	2.26 (0.17)	0.153 (0.005)	5.28 (0.05)	1.14 (0.03)
native	514	0.500 (0.013)	0.105 (0.005)	0.263 (0.004)	0.69 (0.02)	0.186 (0.002)	2.42 (0.05)	0.051 (0.003)	5.64 (0.09)	0.97 (0.02)
apo[c]	458	0.395 (0.019)	0.209 (0.020)	0.263 (0.017)	0.83 (0.08)	0.248 (0.009)	2.72 (0.13)	0.094 (0.008)	6.88 (0.18)	1.62 (0.07)
apo[c]	514	0.404 (0.018)	0.122 (0.011)	0.201 (0.008)	0.79 (0.05)	0.332 (0.005)	2.79 (0.08)	0.063 (0.008)	6.48 (0.22)	1.55 (0.06)
sq anaer[d]	458	0.634 (0.017)	0.116 (0.007)	0.202 (0.008)	0.61 (0.03)	0.115 (0.003)	2.31 (0.08)	0.049 (0.002)	5.86 (0.09)	0.75 (0.02)
sq aer[e]	458	0.658 (0.030)	0.089 (0.007)	0.172 (0.007)	0.56 (0.03)	0.099 (0.003)	2.19 (0.10)	0.071 (0.003)	5.50 (0.06)	0.76 (0.02)
rec FMN[f]	458	0.241 (0.015)	0.182 (0.019)			0.125 (0.004)	1.74 (0.11)	0.634 (0.005)	5.09 (0.02)	3.49 (0.03)
free FMN	458					0.114 (0.043)	3.08 (0.41)	0.886 (0.044)	5.11 (0.05)	4.88 (0.27)
rec ϕRF[g]	514	0.427 (0.020)	0.115 (0.010)	0.293 (0.007)	0.723 (0.032)	0.208 (0.004)	2.64 (0.10)	0.073 (0.006)	6.25 (0.16)	1.27 (0.05)

[a] Enzyme (17 μM) in 25 mM phosphate buffer, containing 20% glycerol, 0.1% lubrol, 0.1 mM EDTA, and 0.1 mM DTT, pH 7.7, was measured at 10 °C. The data were obtained as described under Materials and Methods. Values in parentheses are the standard errors. [b] Mean lifetimes ($\langle\tau\rangle = \sum_i \alpha_i\tau_i$) must be used for energy-transfer measurements [cf. Kulinski et al. (1987)]. [c] FMN-depleted reductase. [d] Semiquinone under anaerobic conditions. [e] Semiquinone under aerobic conditions. [f] FMN-recombined FMN-depleted reductase. [g] 7,8-Phenylriboflavin-recombined FMN-depleted reductase.

is 4.64×10^{-15} cm^3 M^{-1}. In the case of the semiquinone (not shown), the spectral overlap becomes even larger (overlap integral is 2.01×10^{-14} cm^3 M^{-1}) and energy transfer is only possible between FAD and the nonfluorescent FMN semiquinone.

Fluorescence Lifetimes. The performance of the laser setup was checked by measuring erythrosin B in water from time to time (Visser & Van Hoek, 1988). The compound showed a monoexponential fluorescence decay curve. The recovered lifetime was 75 ps upon excitation at 458 nm at 10 °C and is in good agreement with values published from other laboratories (Rodgers, 1981).

The fluorescence lifetimes of various samples of NADPH–cytochrome P-450 reductase are collected in Table I. Except for the reconstituted FMN-depleted reductase the experimental curves had to be fitted with four exponentials; the use of only three exponentials led to unacceptable fits. The several flavoproteins studied so far by time-dependent fluorescence techniques all exhibit complex decay curves (Visser et al., 1980, 1984a; De Kok & Visser, 1987). A detailed explanation for this behavior of protein-bound flavin is difficult to provide, because the multiexponential decays could be due to a number of different effects that are hard to evaluate quantitatively. Our preparations have been analyzed by various biochemical techniques and found to be homogeneous with respect to cofactor content and free of other proteins. We therefore believe that the complex fluorescence behavior of the reductase cannot be ascribed to heterogeneity of the preparations. A structural heterogeneity of reductase molecules with respect to the flavin environment is another possibility for the observed complex fluorescence behavior of the protein. Such differences can hardly be observed by any technique, except for TCSPC fluorescence spectroscopy, by which even a small fraction of molecules with different fluorescence properties can be observed. In such molecules the flavin would still be strongly bound, the environment of the isoalloxazine moiety of the prosthetic group being altered in a dynamic fashion, the interaction between the flavin and the apoprotein thus being modulated by neighboring amino acid residues or segments of the protein. In a fluctuating environment (on a time scale comparable to the fluorescence lifetime) a distribution of microstates would thus result, and a tri- or tetramodal distribution of lifetimes would be a more appropriate model to describe the fluorescence decay kinetics (Alcala et al., 1987). Since the reductase contains both FMN and FAD, the total fluorescence behavior of the enzyme would be even more complex.

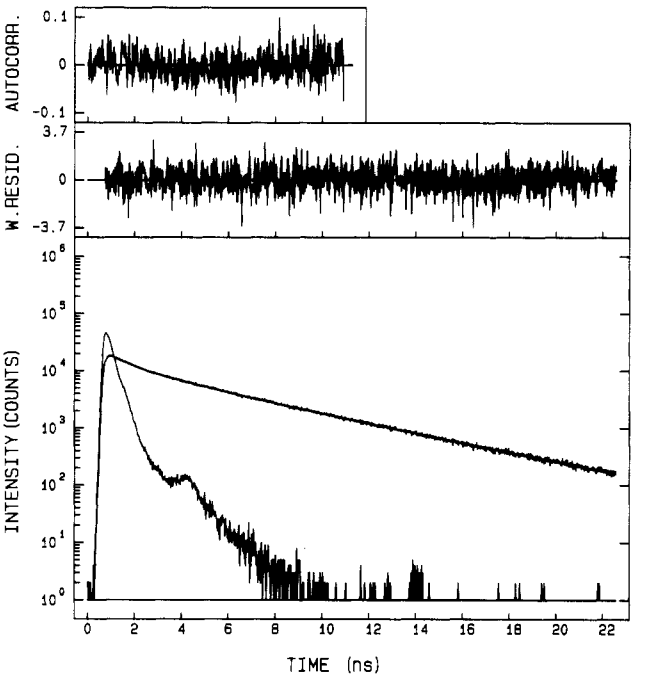

FIGURE 2: Experimental and calculated fluorescence decay curves of native NADPH–cytochrome P-450 reductase (17 μM) in 25 mM potassium phosphate buffer, containing 20% glycerol, 0.1% lubrol, 0.1 mM EDTA, and 0.1 mM DTT, pH 7.7, at 10 °C. The excitation wavelength was 458 nm, and the emission wavelength 531 nm. The response of erythrosin B in water is also given. The weighted residuals and the autocorrelation function of the residuals are also shown. Parameter values are listed in Table I. Statistical parameters are $\chi^2 = 1.0$, Durbin–Watson parameter = 1.98, and number of zero passages in the autocorrelation function = 232 for 990 channels of analysis.

Figure 2 shows an example of the fluorescence decay of native reductase (78 kDa), together with the weighted residuals and the autocorrelation function of the residuals. The latter two curves measure the quality of the fit, and the patterns indicate that fitting the experimental curve with four exponentials leads to a perfect fit. The fluorescence lifetimes thus

Table II: Anisotropy Decay Parameters of Different Samples of NADPH–Cytochrome P-450 Reductase[a]

sample	λ_{ex} (nm)	β_1	ϕ_1 (ns)	β_2	ϕ_2 (ns)	β_3	ϕ_3 (ns)	$r(0)$
native	458	0.189 (0.041)	0.71 (0.11)	0.118 (0.042)	2.1 (0.4)	0.046 (0.002)	65.2[b]	0.353 (0.059)
native	514	0.132 (0.002)	2.76 (0.13)	0.229 (0.002)	65.2[b]			0.361 (0.003)
apo	458	0.156 (0.003)	0.88 (0.05)	0.145 (0.003)	21 (1)			0.301 (0.004)
apo	514	0.160 (0.006)	1.95 (0.12)	0.194 (0.007)	24 (2)			0.354 (0.009)
sq anaer	458	0.159 (0.006)	2.61 (0.16)	0.182 (0.007)	94 (26)			0.341 (0.009)
sq aer	458	0.220 (0.004)	1.71 (0.06)	0.119 (0.004)	58 (11)			0.339 (0.006)
rec FMN	458	0.342 (0.005)	0.43 (0.01)	0.026 (0.003)	5.0 (0.7)			0.368 (0.006)
free FMN	458	0.354 (0.004)	0.461 (0.007)					0.354 (0.004)
rec ϕRF	514	0.153 (0.003)	3.90 (0.19)	0.203 (0.004)	65.2[b]			0.356 (0.005)

[a] See footnotes of Table I for experimental details and abbreviations; values in parentheses are the standard errors. [b] Fixed parameter at fitting.

obtained (Table I) vary from about 0.1 to about 5 ns. The shortest lifetime has a contribution of 50–60% to the total fluorescence decay (α values). The other three lifetimes each contribute about 10%. The FMN-depleted enzyme contains only FAD as prosthetic group, and one is thus tempted to assign the lifetimes observed in the native enzyme to FAD. This interpretation might be correct, but removal of FMN could also influence the conformation of the protein and thereby indirectly the fluorescence properties of bound FAD in more subtle ways than the apparent change in α ratios and, e.g., the considerable lengthening of τ_4. The mean fluorescence lifetime of the semiquinone sample is decreased as compared to the average lifetime of the holoprotein and the FMN-depleted protein. This observation suggests that energy transfer occurs from FAD to the nonfluorescent FMN in the semiquinone state.

Reconstitution of the FMN-depleted enzyme by addition of an excess of FMN and removal of unbound FMN by column chromatography yields a holoenzyme preparation whose cytochrome *c* activity is almost the same as that of the native protein. The fluorescence properties of native and reconstituted enzyme differ greatly however. This was the only sample tested that could be fitted by three exponentials. The rather long mean fluorescence lifetime indicates that FMN in reconstituted FMN-depleted reductase may be bound more weakly than in native reductase. This interpretation is supported by recent ^{31}P NMR results (P. J. M. Bonants and F. Müller, unpublished observations) showing that the recombination is composed of two reactions: a fast, but weak, binding of FMN restoring the activity and a very slow reaction involving probably a conformational change leading to tight binding of FMN.

The fluorescence quantum yield of native reductase was determined to be ca. 7×10^{-4}. Calculating the quantum yield from the observed average lifetime (1.14 ns) and the radiative lifetime of flavins (18 ns; Visser & Müller, 1979), we find $Q_f = 0.063$, which is much higher than the observed quantum yield. Hence, strong static quenching of the flavin fluorescence occurs in the native reductase.

In contradiction to published results (Visser, 1984; Visser et al., 1984b) free FMN was found in this study to exhibit two fluorescence lifetimes (about 10% of a shorter lifetime of 1–3 ns and 90% of a lifetime of 5.1 ns). The contribution of the shorter lifetime component becomes smaller when the buffer is replaced by water alone. This is not a unique property of FMN but is also found with various lumiflavin derivatives (A. J. W. G. Visser, unpublished data).

Anisotropy Measurements. The results of the time-dependent fluorescence anisotropy measurements, using the nonassociative model (which means that all lifetime species are assumed to exhibit identical reorientational behavior), are more informative than fluorescence lifetimes and are, in addition, easier to interprete. The decay parameters are given in Table II. Figure 3 represents experimental results together with the fitted data. Most of the experimental data could be adequately fitted with two exponential functions, the rest requiring three exponentials. In some cases it was necessary to fix the correlation time (65 ns) for overall tumbling of the native protein. Excitation at 514 nm yielded an experimental curve which could be fitted with two exponentials. The excitation wavelength dependent experimental curves (Figure 3) clearly demonstrate that excitation at 458 nm creates a

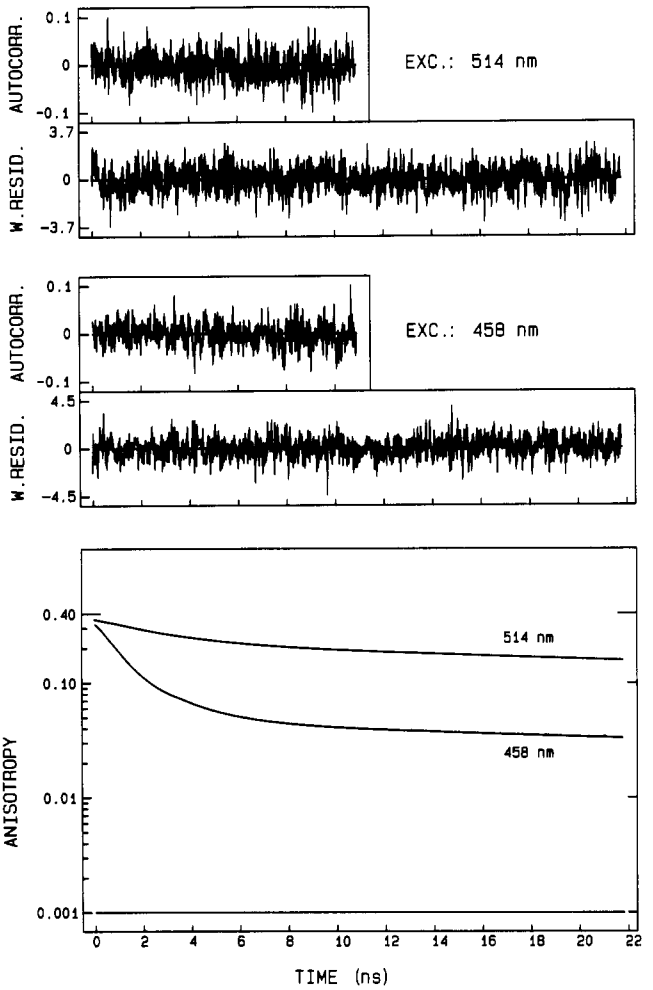

FIGURE 3: Fitted anisotropy decay curves of native NADPH–cytochrome P-450 reductase (17 μM) in 25 mM potassium phosphate buffer, containing 20% glycerol, 0.1% lubrol, 0.1 mM EDTA, and 0.1 mM DTT, pH 7.7, at 10 °C with excitation at 458 nm or at 514 nm. The weighted residuals between experimental and calculated anisotropies and the autocorrelation function of the residuals are also given. Parameters values are listed in Table II. Statistical parameters: (458 nm) $\chi^2 = 1.05$, Durgin–Watson parameter = 1.93, number of zero passages of parallel component = 234 and of perpendicular component = 236; (514 nm) $\chi^2 = 1.09$, Durbin–Watson parameter = 1.95, number of zero passages of parallel component = 252 and of perpendicular component 255; 990 channels of analysis.

component with a short correlation time, which is not observed on excitation at 514 nm.

When the correlation time of tumbling of the protein was a fixed parameter in the fit of the anisotropy decay of the FMN-depleted enzyme, the fit turned out not to be optimal. Upon leaving all the parameters free to readjust, the fit was successful and a long correlation time of about 20 ns was recovered. Upon red-edge excitation of the same sample, the shortest correlation time increased 2-fold, but the limiting anisotropy and long correlation time remained about the same. The large difference in long correlation times between the FMN-depleted enzyme and holoenzyme is probably due to segmental motion or a different orientation of the transition moment of FAD relative to the protein matrix upon removal of FMN. It is highly probable that cytochrome P-450 reductase is not spherically symmetric and consequently is not an isotropic rotor. In this case the recovered correlation times of tumbling are influenced by the relative orientation of the emission transition moments in the protein.

The one-electron-reduced reductase showed two correlation times, a rather long one, which is about the same as the rotational correlation time of the protein, and one of about 2 ns. The latter value was also found with all other samples. It is interesting to note that longer correlation times are obtained under anaerobic rather than under aerobic conditions. Although the semiquinone of the reductase reacts only slowly with molecular oxygen, the decrease in correlation times is due to the presence of some oxidized FMN in the reductase sample, leading to increased depolarization. This is also reflected by the total fluorescence anisotropy, $r(\text{tot})$, calculated from the parameters of the fluorescence decay (eq 1) and fluorescence anisotropy decay (eq 2) according to

$$r(\text{tot}) = \int_0^\infty I(t)\, r(t)\, dt \Big/ \int_0^\infty I(t)\, dt \qquad (20)$$

$r(\text{tot})$ of the semiquinone sample decreases from 0.26 to 0.20 when air is admitted to the sample. Concomitantly with the decrease in the correlation times, the amplitude of the shortest component increases.

Reconstitution of FMN-depleted reductase yields correlation times that are very different from those observed with the native protein, even though the reconstituted sample, measured about 1 h after reconstitution, exhibited an activity of about 95% of that of the native protein. The longer correlation time is only about 10 times longer than that of free FMN. From this it is concluded that FMN reorients more freely in the reconstituted sample than in the native protein but is still bound to it, and its degree of reorientational freedom is also greater as indicated by the corresponding amplitudes (β values, Table II). The presence of free FMN is unlikely because the sample of the reconstituted protein was chromatographed prior to the measurements, thus removing unbound FMN from the protein.

When 7,8-phenyl-10-ribitylisoalloxazine (Bonants, 1987) was used as a prosthetic group, rotational correlation times that are almost identical with those of native protein excited at 514 nm (Table II) were observed. The similar values for the two proteins suggest that full recombination had occurred at the time of the fluorescence measurements. Furthermore, it is emphasized that the light absorption spectrum of this model compound is red shifted and that therefore this compound is preferentially excited at 514 nm so that it can be used as a reporter group for the FMN binding site.

Steady-State Fluorescence Anisotropy. The steady-state wavelength-dependent anisotropies of native and FMN-depleted NADPH–cytochrome P-450 reductase are shown in

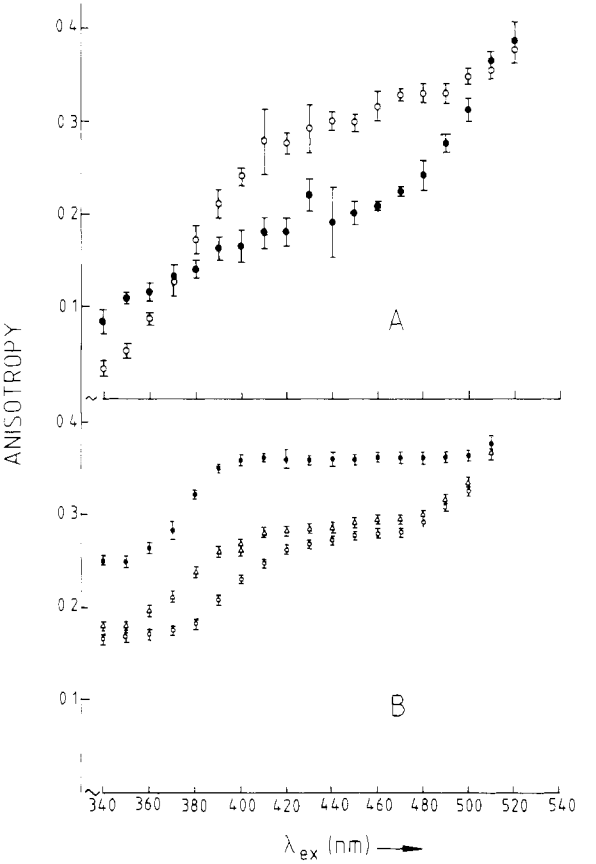

FIGURE 4: Steady-state fluorescence anisotropy of native (●) and FMN-depleted (○) NADPH–cytochrome P-450 reductase 25 mM potassium phosphate, containing 20% glycerol, 0.1% lubrol, 0.1 mM EDTA, and 0.1 mM DTT, pH 7.7 (A), and of methyllumiflavin (MLF) (●), MLF-$(CH_2)_3$-MLF (△), and MLF-$(CH_2)_6$-MLF (○) in poly(methyl methacrylate) (B) as a function of the excitation wavelength (λ_{ex}). The temperature was 10 °C.

Figure 4A. Both preparations exhibit the expected wavelength-dependent anisotropy for the two lowest transitions of the flavin molecule, except that the anisotropy for the native enzyme is lower in the 400–500-nm region than that of the FMN-depleted form. The values at 520 nm are the same for both preparations and agree well with the initial value (0.36 ± 0.01, see Table II) observed by the time-dependent anisotropy measurements.

As a model system for the NADPH–cytochrome P-450 reductase we investigated N^3-methyllumiflavin and $N^{10},N^{10\prime}$-trimethylene- and $N^{10},N^{10\prime}$-hexamethylenebis-(3,7,8-trimethylisoalloxazine) immobilized in poly(methyl methacrylate), their fluorescence lifetime and time-dependent anisotropy properties having been determined earlier (Visser et al., 1983). The steady-state anisotropy curves of these compounds are shown in Figure 4B. The steady-state anisotropy of the monomeric compound across the first transition is 0.35 ± 0.01. The corresponding values for the dimeric flavin molecules are lower and differ from each other. Of note, however, is that the steady-state anisotropies of the dimeric flavins increase at wavelengths longer than 480 nm and reach that of the monomeric flavin at about 510 nm.

Discussion

The decays of the fluorescence emission of the various forms of NADPH–cytochrome P-450 reductase examined in this work are composed of multiple relaxations (Table I). We found that the experimental data could only adequately be fitted by four, or in one case by three, exponential decay

Table III: Calculated Second-Rank Order Parameters (S) and Diffusion Coefficients ($D_\perp$) of the Cofactors in the Reductase[a]

sample	λ_{ex} (nm)	flavin[b] monitored	A_∞[c]	S	θ_0[d] (deg)	ϕ_{eff}[e] (ns)	$D_\perp$ (ps^{-1})
apo	458	FAD	0.145 (0.003)	0.694 (0.009)	38.8 (0.6)	0.92 (0.05)	94 (6)
apo	514	FAD	0.194 (0.007)	0.740 (0.017)	35.4 (1.3)	2.1 (0.1)	36 (3)
sq	458	FAD	0.182 (0.007)	0.731 (0.017)	36.1 (1.3)	2.7 (0.2)	29 (2)
rec ϕRF	514	7,8ϕ-riboflavin	0.203 (0.004)	0.755 (0.009)	34.2 (0.7)	4.2 (0.2)	17 (1)
native	514	FAD + FMN	0.229 (0.002)	0.796 (0.005)	31.0 (0.4)	2.9 (0.1)	21 (1)

[a] See footnotes of Table I for abbreviations and text for details; values between parameters are the standard errors. [b] Fluorescence originates from this prosthetic group. [c] Limiting anisotropy at long time compared to the fluorescence lifetime. [d] Cone half-angle for restricted fluorophore motion. [e] Effective correlation time of restricted reorientation motion.

components. This interpretation should be considered as a minimal model. Since the observed heterogeneity of the fluorescence emission of the complex protein could be caused by different mechanisms, it is difficult to assign the observed relaxations to a particular effect. It is now well accepted that biomolecules are not static molecules but undergo dynamic structural changes in solution. The dynamic changes of interest here consist of spatial displacements of amino acid residues in the immediate neighborhood of the isoalloxazine moiety of the prosthetic groups or of structural fluctuations of segments or domains of the protein. This probably causes the fluorescence to appear heterogeneous. Hence, a tri- or tetramodal distribution of lifetimes is observed rather than separate lifetimes attributable to distinct states of the structure of the protein. From this it is obvious that one must be cautious with a detailed interpretation of the experimental data. Despite this handicap it is still possible to extract some interesting information from time-dependent fluorescence data, especially from anisotropy data.

An interesting point is the wavelength dependence of the correlation times found in the anisotropy decay. Excitation of native reductase at 514 nm instead at 458 nm yields two correlation times instead of three, and the steady-state anisotropy increases (from 0.13 to 0.29), the shortest correlation time disappearing from the decay curve (Figure 3). The much shorter correlation times and lower limiting anisotropy at long times observed in FMN-depleted reductase as compared to those of the native protein suggest that the binding interaction between FAD and the protein is altered or some structural changes occur during the preparation of the FMN-depleted reductase.

The correlation times of the reductase in the semiquinone state under anaerobic and aerobic conditions also differ (Table II). This difference can be ascribed to the presence of a few percent of fully oxidized reductase in the sample investigated under aerobic conditions, as ascertained by spectrophotometric measurements of the two samples.

Reconstitution of FMN-depleted reductase yielded very short correlation times (Table II), even though the samples used were gel chromatographed immediately prior to the experiments. In addition, activity measurements indicated that the samples were 90–95% active as compared to native protein. Therefore, we can conclude that FMN in reconstituted FMN-depleted reductase is bound differently than in the native protein. This suggests that the activity as measured by the reduction of the artificial electron acceptor cytochrome c alone is not a particularly reliable indicator that the binding interaction between FMN in native and reconstituted FMN-depleted reductase is the same. In fact, as already mentioned above, the recombination process between FMN and FMN-depleted reductase restoring the original conformation of the native protein is slow.

The wavelength-dependent time-resolved and static anisotropy data of native and FMN-depleted reductases (Table II, Figures 3 and 4) strongly indicate that energy transfer occurs between the two flavins in the protein. This is supported by the data obtained from the air-stable semiquinone of the reductase under anaerobic and aerobic conditions. Under anaerobic conditions, where only one flavin (i.e., FAD) is fluorescent, the total anisotropy is about the same as that observed from native and FMN-depleted reductase on excitation at 514 nm. The possible occurrence of energy transfer between the two flavins in the reductase is more obvious from the steady-state anisotropy data (Figure 4A), showing that the anisotropy in the excitation region 420–480 nm is smaller for native than for FMN-depleted reductase.

To corroborate the idea of energy transfer in the reductase, we investigated model systems in the immobilized state mimicking our protein system. The steady-state anisotropy of monomeric N^3-methyllumiflavin under these conditions is larger than those of the dimeric flavins (Figure 4B), similar to the observations made with native and FMN-depleted reductases (Figure 4A). A feature common to both sets of experiments is that the steady-state anisotropies at about 514 nm become equal (Figure 4). The time-dependent anisotropies of the model systems [cf. Figure 3 in the paper of Visser et al. (1983)] and the reductase samples also show great similarities; i.e., the dimeric flavins and native reductase exhibit a fast initial decay of the anisotropy upon excitation in the main part of the absorption band. Since it has been demonstrated that energy transfer occurs in the model systems (Visser et al., 1983), we can now state that this is also the case in the native reductase on the basis of the great similarities of both sets of data.

By use of the formalism described under Materials and Methods, the rate constant of energy transfer (k_T) is 0.70 ± 0.11 ns and the angle between the optical transition moments of FAD and FMN can be obtained from anisotropy measurements at 458 nm, by using the results obtained at 514 nm, which, on the other hand, give the correlation time of restricted motion of the flavins and the second-rank order parameter S.

Dale et al. (1979) have provided contour plots of minimum and maximum values of κ^2 for donor–acceptor pairs reorienting in uniaxial potentials. The relevant parameters for an estimation of the boundaries of κ^2 are the axial and transfer depolarization factors, which can be obtained from the time-resolved anisotropy data of the various samples as indicated in the section on the interpretation of the time-dependent anisotropy data.

The second-rank order parameter or axial depolarization factor can be obtained from the limiting anisotropy at long time (A_∞) as described by Van der Meer et al. (1984). The results are shown in Table III together with the calculated half-angle of a cone within which the probes can reorient randomly (Lipari & Szabo, 1980). Values between 0.70 and 0.80 were obtained for the axial depolarization factors (or second-rank order parameters) of the flavins.

From the preexponential amplitude of the shortest anisotropy component of the native enzyme excited at 458 nm, the transfer depolarization factor (−0.07 ± 0.29) can be obtained.

The corresponding angle Θ_T between the optical transition moments in FAD and FMN was found to be 58 ± 25°. Given the large uncertainty in the transfer depolarization factor, it implies that the interflavin angle cannot be determined accurately. From the initial anisotropy $r(0)$ the angle between absorption and emission transition moments in a flavin molecule can be calculated. For free FMN this angle was found to be 16.1 ± 0.2°.

From the order parameter and boundaries of κ^2 upper and lower limits of the distance R between FAD and FMN in the native reductase can be calculated. This distance was found to be 1.6–2.5 nm. The errors in these limits, estimated for the most unfavorable situation (high order parameter and a 10% error in the overlap integral), amounted to 0.05 and 0.08 nm and thus could be safely omitted. A similar calculation was performed for the semiquinone sample. Although the uncertainty in κ^2 is large, because no information is available on the transfer depolarization factor and axial depolarization factor of FMN semiquinone, the obtained distance is similar to that in the native enzyme [(2.0 ± 0.6)–(3.5 ± 0.1) nm]. In the latter calculation it was assumed that $P_2(\mathbf{D}_1^x \cdot \mathbf{D}_2^x)$ takes up its limiting values of 0 and 1 corresponding to limits on κ^2 of 0 and 4. Assuming that there is no gross conformational change upon reduction of FMN, we can therefore tentatively conclude that the limits of the distance between the isoalloxazines of FAD and FMN are between 2.0 ± 0.1 and 2.5 ± 0.1 nm.

Although many dimeric flavoproteins are known, there is no evidence that electrons, as in NADPH–cytochrome P-450 reductase, use one flavin as the entry port and the other as the exit port. Recently, the three-dimensional structure of flavocytochrome b_2 from bakers' yeast at 0.24-nm resolution has become available (Mathews & Xia, 1987; Lederer & Mathews, 1987). This flavoprotein contains, besides the flavin, a heme group as electron carrier. The enzyme exhibits a similar electron-transfer mechanism as NADPH–cytochrome P-450 reductase, and the crystallographic study showed that the two prosthetic groups are separated by 1.6 nm. The distance between FAD and FMN in NADPH–cytochrome P-450 reductase determined in the present study is calculated to be ~2 nm, suggesting that reasonable distances can be calculated from such fluorometric data, even though a number of fairly critical assumptions had to be made. Our data are also in good agreement with theoretical estimates suggesting that even a 2.8-nm separation between two redox centers is small enough for efficient electron transfer (Kuki & Wolynes, 1987).

In conclusion, it has been shown that time-dependent and steady-state fluorescence measurements can yield useful information regarding the distance between two like chromophores in one protein. Our results and those published by others show that the distance between two protein-bound chromophores involved in electron-transfer reactions can be surprisingly large, indicating that a direct interaction between these chromophores is not a requirement for efficient electron transfer between them. The next question to be addressed concerns the mechanism by which the electrons are efficiently and selectively transferred between the two redox centers in such proteins.

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Cysteinyl Peptide Labeled by 3-Bromo-2-ketoglutarate in the Active Site of Pig Heart NAD^+-Dependent Isocitrate Dehydrogenase†

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ABSTRACT: The substrate affinity label 3-bromo-2-ketoglutarate (BrKG) reacts covalently with pig heart NAD^+-specific isocitrate dehydrogenase with complete inactivation and incorporation of about 0.8 mol of reagent/mol of average enzyme subunit [Bednar, R. A., Hartman, F. C., & Colman, R. F. (1982) *Biochemistry 21*, 3681–3689]. Protection against inactivation is provided by isocitrate and Mn^{2+}. We have now identified a critical modified peptide by comparison of the peptides labeled by BrKG at pH 6.1 in the absence and presence of isocitrate and Mn^{2+}. Modified enzyme, isolated from unreacted BrKG, was incubated with [^{3}H]$NaBH_4$ to reduce the keto group of protein-bound 2-ketoglutarate and thereby introduce a radioactive tracer into the modified amino acid. Following carboxymethylation and digestion with trypsin, the specific modified peptide was isolated by reverse-phase HPLC, first in 0.1% trifluoroacetic acid with a gradient in acetonitrile and then in 20 mM ammonium acetate, pH 5.8, with an acetonitrile gradient. Gas-phase sequencing gave the modified peptide: Ser-Ala-X-Val-Pro-Val-Asp-Phe-Glu-Glu-Val-Val-Val-Ser-Ser-Asn-Ala-Asp-Glu-Glu-Asp-Ile-Arg. The corresponding tryptic peptide that was isolated from unmodified enzyme yielded the same sequence except for (carboxymethyl)cysteine at position 3, suggesting that cysteine is the target of 3-bromo-2-ketoglutarate. Pig heart NAD^+-dependent isocitrate dehydrogenase is composed of three distinct subunits (α, β, and γ) that can be separated by chromatofocusing in urea and identified by analytical gel isoelectric focusing. The peptide modified by 3-bromo-2-ketoglutarate, which is in or near the substrate site, is derived only from the separated γ subunit.

The pig heart NAD^+-specific isocitrate dehydrogenase [*threo*-D_S-isocitrate:NAD^+ oxidoreductase (decarboxylating), EC 1.1.1.41] has been shown to be inactivated by covalent reaction with 3-bromo-2-ketoglutarate (BrKG),[1] which acts as an affinity label of the isocitrate binding site (Bednar et al., 1982a). A marked decrease in the rate constant for in-

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[1] Abbreviations: BrKG, 3-bromo-2-ketoglutarate; Mes, 2-(*N*-morpholino)ethanesulfonic acid; DTT, dithiothreitol; Cm, carboxymethyl; PTH, phenylthiohydantoin.

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