FLAVIN-PROTEIN INTERACTIONS IN FLAVOENZYMES. STUDIES OF FMN BINDING IN AZOTOBACTER FLAVODOXIN USING PROTEIN FLUORESCENCE LIFETIME MEASUREMENTS†

Leonard J. Andrews, Martha L. MacKnight, Jack Ryan and Gordon Tollin*

Department of Chemistry
University of Arizona
Tucson, Arizona 85721

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SUMMARY

Frequency doubling of a nitrogen laser-pumped Rhodamine G dye laser has been used to provide an excitation pulse for the measurement of the tryptophan fluorescence decay of Azotobacter flavodoxin, apoflavodoxin and N-bromosuccinimide (NBS) oxidized derivatives. The fluorescence decay of the apoprotein can be resolved into two components. The major contribution (~95%) has a lifetime of 4 nsec; the minor emission has a decay time of 1 nsec. The holoprotein fluorescence decay is non-exponential with lifetime components close to 1 nsec. These data, along with results obtained with a series of NBS oxidized derivatives, are interpreted in terms of the presence of a highly fluorescent tryptophan in the apoprotein which is strongly quenched upon FMN binding and which probably is part of the coenzyme binding site.

The flavodoxins, because of their relative simplicity and stability, are proving to be particularly useful systems for the investigation of flavin-protein interactions (1, 2, 3, 4). Studies of tryptophyl and FMN fluorescence quenching have provided kinetic and equilibrium constants for flavin binding (4, 5, 6). Fluorescence yield measurements of the holo and apoproteins (6), as well as yield measurements of apoprotein in which some of tryptophan side chains have been oxidized (7), indicate a close association of FMN with one tryptophan side chain in the coenzyme binding site. This has been shown to be the case in the flavodoxin from D. vulgaris by x-ray crystallography (8). In addition, there is kinetic and spectroscopic evidence to suggest that some protein structural changes occur during coenzyme binding (3, 4). Inasmuch as excited state processes in the flavodoxin from Azotobacter vinelandii have been well characterized using steady state techniques (6), we felt that it would be useful to investigate the time

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*To whom reprint requests should be addressed.

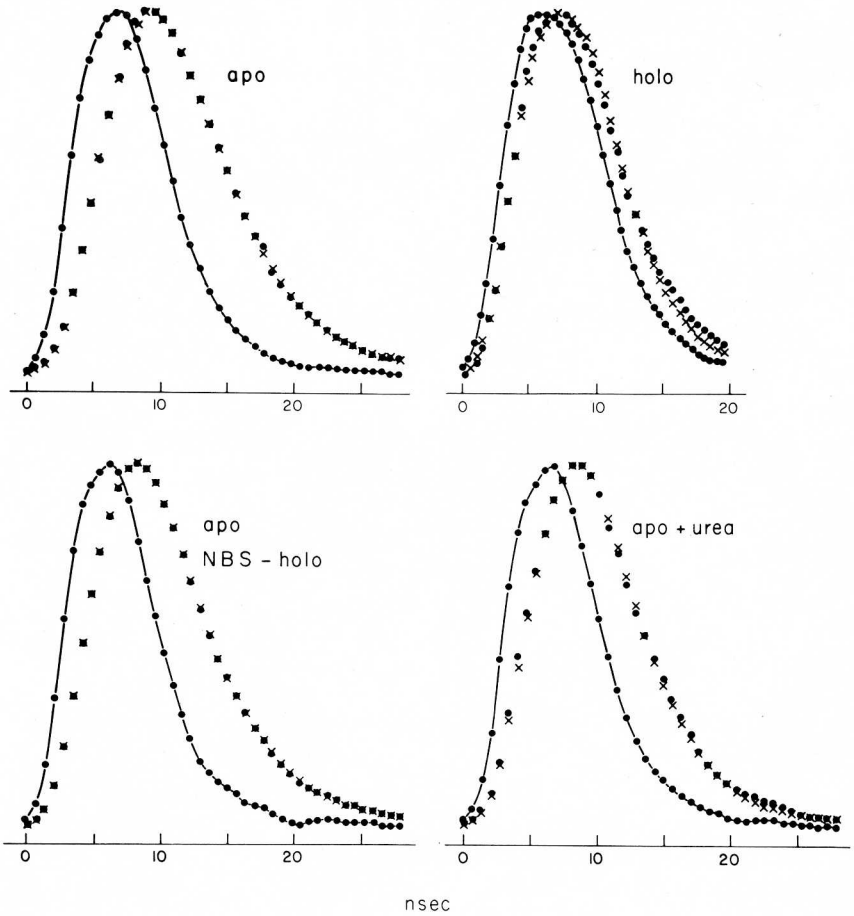
resolved fluorescence parameters. The similarities which exist between the Azotobacter and D. vulgaris flavodoxins (3, 9) make such a study of particular interest.

MATERIALS AND METHODS

The preparation of holoprotein, apoprotein, and N-bromosuccinimide (NBS) oxidized derivatives has been described previously (5, 7). The basis for the fluorescence lifetime instrument was a pulsed UV laser excitation source. A molecular nitrogen laser (338.1 nm) transversely pumped a Rhodamine-6G dye laser. The dye laser output was tuned to approximately 590 nm and focused into a lithium formate monohydrate crystal to generate its second harmonic. The resulting 7 nsec (fwhm) pulse at approximately 295 nm excited the protein and fluorescence was detected at right angles with a 1P28 photomultiplier. The output of the photomultiplier went to Tektronix 661 sampling oscilloscope and then to a Mnemotron CAT 400B. The ramp associated with the horizontal sweep of the 661 oscilloscope was used to initiate appropriate trigger pulses for the CAT to insure a one-to-one correspondence of channels. 150 sweeps were averaged. In several experiments, the photomultiplier output was displayed on a Tektronix 7904 real time oscilloscope (500 MHz bandwidth), and recorded on Polaroid film.

RESULTS

In Fig. 1 are shown typical fluorescence decay curves for holoprotein and several derivatives. The set of curves for each protein shows the profile of the exciting laser pulse, the resulting fluorescence pulse, and the calculated fit to the fluorescence pulse. Table 1 lists the calculated decay constants and the relative quantum yields (3, 7) for holoprotein, native apoprotein, denatured apoprotein, and oxidized apoprotein obtained by either direct oxidation of the apoprotein or oxidation of the holoprotein followed by conversion to the apoprotein (7). All decays were sufficiently fast so that deconvolution techniques were required to extract the decay parameters. We found that the most satisfactory approach to this problem was the convolution of trial decay functions with the experimental excitation pulse to fit the observed fluorescence



LEGEND FOR FIGURE

Fluorescence decay properties of Azotobacter flavodoxin and several analogs. Solid lines are laser decay pulse. Points represent experimental (•) and theoretical (x) fluorescence decay curves.

decay. The minimum sum of squared deviations was used as the criterion of best fit and the corresponding root mean squared deviations (RMSD) are listed in Table 1.* All excitation was done at 295 ± 2 nm in air saturated buffer (0.025 M phosphate, pH 7.0).

Because of the high degree of correlation between the fitted parameters, τ_2 and A_1/A_2 must be viewed as qualitative only. However, τ_1 is probably accurate inasmuch as it is responsible for the major portion of the decay in most of the

*The RMSD is computed after first normalizing the experimental and calculated fluorescence pulses to a peak intensity of unity.

TABLE 1

FLUORESCENCE LIFETIME ANALYSIS OF *AZOTOBACTER* FLAVODOXIN AND ITS ANALOGS*

	SINGLE EXPONENTIAL		TWO EXPONENTIALS					
PROTEIN	τ	RMSD	A_1	τ_1	A_2	τ_2	RMSD	ϕ_R
Holo	1.0	0.0319	----------					0.1
Apo	3.8	0.0084	0.81	4.0	0.19	1.0	0.0058	1.0
Apo, NBS holo (1.1 trp)	3.3	0.0141	0.56	4.0	0.44	1.4	0.0052	0.5
Apo, NBS holo (1.6 trp)	3.1	0.0224	0.30	5.1	0.70	1.9	0.0087	~0.1
Apo, NBS (1.1 trp)	3.8	0.0060	0.83	4.0	0.17	2.3	0.0055	0.5
Apo, NBS (1.9 trp)	4.0	0.0121	----------					~0.1

*RMSD = root mean squared deviation

A_1/A_2 = relative contribution of emitting species

ϕ_R = relative emission quantum yield

measurements and this same value was found to recur in the decays of the apoprotein derivatives.

DISCUSSION

In order to interpret fluorescence lifetime data on systems as complex as proteins, it is helpful to consider complementary quantum yield measurements (6, 7). Removal of the FMN prosthetic group from the native holoprotein results in a stable apo form which has a ten-fold greater tryptophan fluorescence quantum yield. NBS oxidation of the apoprotein reduces the yield to approximately that of the holoprotein and oxidizes approximately 2 of the 4 tryptophans present.

Analysis of these data show that only one of the tryptophans is responsible for the reduction of yield. Concomitant with the loss of tryptophan fluorescence is a loss of FMN binding ability. This has also been shown to be a result of the modification of a single tryptophan residue. CD measurements show that some protein conformational changes accompany the oxidation. NBS treatment of the holoprotein, which is then converted into the apo form, produces essentially identical results, i.e., approximately 2 tryptophans are oxidized, fluorescence intensity is reduced by approximately 90%, FMN is released during oxidation and small conformational changes occur. It is important to note that far UV CD measurements indicate that the protein conformations reached upon NBS oxidation of apo and holoproteins are not the same.

Analysis of the fluorescence decay of the apoprotein shows that the emission is dominated by a 4.0 nsec component with a minor contribution from a faster decaying component (approximately 1.0 nsec). The results obtained upon NBS oxidation of the apoprotein provide evidence that the longer lived component is associated with a single tryptophan, and thus one or more of the remaining three tryptophans must be responsible for the faster, less intense decay. Calculation of relative quantum yields ($\phi_R = A_1\tau_1/A_2\tau_2$) from the decay parameters show that the major component accounts for ~95% of the apoprotein fluorescence intensity. This is in good agreement with the steady state value of 90% and lends credence to the deconvolution analysis.

An alternative interpretation of the complex decay is that both components of the emission are due to a single tryptophan which simultaneously emits from two close lying singlet levels. This implies that emission from thermally non-relaxed states is occurring, since rapid establishment of a Boltzman population distribution leads to simple exponential decay. However, in systems in which indolyl dual emission has been established (10), thermal equilibration obtains and exponential decay is observed. Furthermore, in such systems the emission band shape is found to be invariant to exciting wavelength. We feel it to be highly improbable that thermal relaxation in proteins is sufficiently slow com-

pared with bulk solvent environments so as to make fluorescence decay a competitive process, and therefore reject dual emission as the origin of the multiple exponential decay.

When FMN binds to the apoprotein, the 4.0 nsec decay is abolished and only the faster component remains. The data indicate that a single exponential decay may be a poor approximation due to the large RMSD. However, it was not feasible for us to meaningfully decompose this decay curve into components, since this necessitated a signal-to-noise ratio we could not achieve with such fast decays. Clearly, binding of FMN strongly quenches the dominant tryptophan emission in the apoprotein. On the basis of lifetime measurements alone, we cannot distinguish between the possibilities of complete quenching of the highly fluorescent tryptophan, and partial quenching with a concomitant reduction of lifetime to approximately 1 nsec. However, if the latter case obtained, we would not expect a 90% reduction in emission quantum yield, but only approximately a 75% reduction. The yield data thus appear consistent with the interpretation that fluorescence from the dominant tryptophan is completely or nearly completely quenched and that the remainder of the luminescence intensity is due to weak emitters further removed from the FMN binding site. The non-exponential decay of the holoprotein is easily understood in terms of environmental heterogeneity of the emitting tryptophans, as well as the possibility of distance dependent resonance energy transfer to FMN.

Oxidation of 1.1 tryptophan residues in the holoprotein, followed by conversion to the apo form, yielded a highly non-exponential fluorescence decay which is well resolved into two components of 4.1 and 1.4 nsec with approximately equal intensities. This is in accord with a 50% reduction of yield relative to the unmodified apoprotein and shows that a substantial destruction of the highly fluorescing tryptophan residue occurred. When 1.6 tryptophans were oxidized, a further reduction of the intensity of the long-lived tryptophan emission was observed and the shorter-lived component became dominant. At this point in the oxidation, the fluorescence yield is 10-20% that of the apoprotein. The life-

time data clearly show that various tryptophans are oxidized at differing rates and that the oxidation of the highly fluorescent tryptophan is more facile.

The lifetime data also demonstrate that oxidation of the apoprotein follows a different course than does the holoprotein. With 1.1 tryptophans oxidized, the fluorescence decay is rather well fitted by a single exponential with a 3.8 nsec lifetime. Analysis of the decay in terms of two exponentials improves the fit slightly and indicates the possible presence of a minor short lived component, although this resolution is not certain. When 1.9 tryptophans were oxidized, the yield was only approximately 10% of the apo form, yet the decay analysis yielded the same result. It appears therefore that the shorter-lived component is preferentially abolished, in contrast to the finding when the holoprotein is treated with NBS. This is consistent with the far UV CD results mentioned above.

CONCLUSIONS

The fluorescence yield data and the lifetime results show that FMN quenching of the tryptophan residue which dominates the apoprotein fluorescence is rather complete. This is in full agreement with the presence of a highly fluorescent tryptophan in the apoprotein which is (or becomes) part of the FMN binding site and is thus in close proximity to the isoalloxazine ring of the flavin. Such a conclusion is, however, not completely unequivocal inasmuch as protein conformation changes have been shown to accompany coenzyme binding (4) and NBS oxidation (7). Thus, it is possible that both the loss of FMN binding ability upon NBS treatment and the quenching of protein fluorescence upon FMN binding to apoprotein are indirect effects mediated by these protein conformational changes. This type of uncertainty is, of course, a common feature of all chemical modification studies. Although further work will be required to allow a definitive choice to be made between these alternatives, the x-ray results obtained with the D. vulgaris flavodoxin and the similarities which exist between this protein and the Azotobacter flavodoxin would certainly suggest that the binding site tryptophan model is the most likely possibility.

However, from the fluorescence data it is not possible to infer that FMN and the emitting tryptophan are in actual Van der Waals contact with each other, since resonance energy transfer and exchange interactions totally dominate fluorescence decay within about one molecular diameter.

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