protein interior where both rapid oscillations and longer time processes are expected to contribute to the relaxation. As the time scale separation between the internal rotations and tumbling increases, the independent lattice jump model provides a poorer description of the NMR relaxation and improved models are required (7).

In summary, the present work provides a theoretical foundation for the continuing effort to obtain information about the internal dynamics of proteins from NMR measurements.

Supported in part by grants from the National Science Foundation and the National Institutes of Health. R. M. Levy is a National Institutes of Health Postdoctoral Fellow.

Received for publication 29 December 1979.

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PROTON NUCLEAR MAGNETIC RESONANCE AND FLUORESCENCE SPECTROSCOPIC STUDIES OF SEGMENTAL MOBILITY IN AEQUORIN AND A GREEN FLUORESCENT PROTEIN FROM *AEQUOREA FORSKALEA*

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Aequorin is a protein of low molecular weight (20,000) isolated from the jellyfish *Aequorea forskalea* which emits blue light upon the binding of Ca^{2+} ions (for review, see reference 1). This bioluminescence requires neither exogenous oxygen nor any other cofactors. The light emission occurs from an excited state of a chromophore (an imidazolopyrazinone) which is tightly and noncovalently bound to the protein. Apparently the binding of Ca^{2+} by the protein induces changes in the protein conformation which allow oxygen, already bound or otherwise held by the protein, to react with and therein oxidize the chromophore. The resulting "discharged" protein remains intact, with the Ca^{2+} and the chromophore still bound, but is incapable of further luminescence. The fluorescence spectrum of this discharged protein and the bioluminescence spectrum of the original "charged" aequorin are identical. A green

fluorescent protein (GFP) of ~30,000 mol wt isolated from the same organism, functions in vivo as an acceptor of energy from aequorin and subsequently emits green light. We are applying proton nuclear magnetic resonance (NMR) spectroscopy and fluorescence spectroscopy to examine structural details of, and fluctuations associated with the luminescent reaction of aequorin and the in vivo energy transfer from aequorin to the GFP.

Proton NMR experiments were performed on multiply exchanged D_2O solutions of aequorin in phosphate buffers. The protein concentrations were 2–3 mM. The HDO signal was eliminated by presaturation. The temperature in the experiments was 10°–15°C. Chemical shifts given below are with reference to DSS (dimethyl silapentane sulfonate) as internal standard. The experiments were performed on a 360-MHz Nicolet NMR spectrometer at the Purdue University Biochemical Magnetic Resonance Laboratory.

The C(2) protons from the 4 (or 5) histidine residues (2) in calcium bound aequorin resonate ~8.6 ppm at pH 5.7 and ~7.7 ppm at pH 10.5. At intermediate pHs (e.q., pH 8.0), the signals separate from each other. The pk_a values range between 6.7 and 7.4 for the different histidines. The resonances associated with some of the aromatic protons on the chromophore were assigned on the basis of a spectrum of synthetic chromophore in CD_3OD (the chromophore is not soluble in water). Some of the chromophore signals shift by ~0.4 ppm on the protein. The aromatic proton resonances of the six tyrosines appear to superpose to give two doublets at ~6.9 ppm and 7.2 ppm (at pH 8.0). These resonances shift with pH with a pk_a of ~10.5. The relationship between the tyrosine doublets (as well as the two doublets of the parahydroxyphenyl moiety of the chromophore) was established by homonuclear proton spin decoupling.

Aequorin is also known to bind various rare earth ions and luminesce. The proton NMR spectrum of aequorin bound with Eu^{3+} displays some drastic changes with respect to the spectrum of calcium-bound aequorin indicating the potential value of the lanthanide ions as structural probes of this protein. Mg^{2+} ions act as antagonists for aequorin, probably binding at or near the Ca^{2+} or lantanide sites but not inducing the structural change necessary for light emission.

Spin-lattice relaxation (T_1) measurements were made on solutions of metal free aequorin and aequorin bound with Ca^{2+} and Mg^{2+}. In all cases the aromatic and aliphatic protons seem to relax as two separate groups. An isolated chromophore signal ~8.5 ppm assigned to the pyrazine proton relaxes slower than all other protons. Measurements made so far yield the following T_1 values respectively for the four groups of protons, (*a*) the pyrazine proton on the chromophore, (*b*) the histidine protons, (*c*) the aromatic protons of the protein and the chromophore, and (*d*) the aliphatic protons: metal free aequorin (pH 5.8) 1.7*s*, 1.5*s*, 1.5*s*, 0.7*s*; Ca^{2+} aequorin (pH 7.25) 2.9*s*, 0.9*s*, 0.65*s*, 0.6*s*; and Mg^{2+} aequorin, 1.35*s*, 1.3*s*, 1.4*s*, 0.6*s*. The aliphatic protons thus relax twice as fast as the remaining protons in metal-free aequorin and Mg^{2+} aequorin. In Ca^{2+} discharged aequorin the aromatic proton relaxation is accelerated to become close to that for aliphatic protons, but the pyrazine proton relaxation is significantly slowed down.

In aequorin, binding of the chromophore in the native protein is independent of metal ion binding, although in blue fluorescent protein the chromophore is readily dislodged if Ca^{++} ions are sequestered by chelation with EDTA. Fluorescence anisotropy measurements reveal that at 25°C the measured anisotropy for excitation of blue fluorescent protein in the 0–0 band is marginally less than the r_0 for the protein embedded in a rigid glass (glycerol at −60°C). Consequently the Perrin plot (of steady state anisotropy vs. viscosity) shows a low slope and an extrapolated r_0 value identical to the measured r_0 (0.43). Similar data are

obtained with the green fluorescent protein. These data are sufficient to suggest conformational rigidity of both proteins, but other evidence is adduced from: (*a*) the relative insensitivity of aequorin blue fluorescence to oxygen quenching (bimolecular quenching constant [k_{bm}] 0.09 M^{-1} s^{-1}) which is comparable to the lower values observed by Lakowicz and Weber (3) in the study of oxygen quenching of proteins. The green protein fluorescence is essentially insensitive to the quenching effects of oxygen ($k_{bm} < 0.004$ M^{-1} s^{-1}). (*b*) Fluorescence lifetimes and anisotropies are essentially invariant across the emission band and hence reveal no evidence of dipolar relaxation around the chromophore in the green fluorescent protein; and there is only marginal dipolar relaxation in aequorin. (*c*) The green fluorescent protein is denatured by sodium dodeceylsulfate and guanidinium chloride only under harsh conditions and is markedly resistant to proteolytic cleavage by most commonly proteases.

These data, taken together show that the chromophore in Ca^{++} discharged aequorin is rigidly bound; in the green fluorescent protein not only is the chromophore very tightly bound, but the entire protein appears to be markedly conformationally inflexible.

Supported in part by U. S. Public Health Service grant HL 12186, The Mayo Foundation, and a grant from the Research Corporation. The NMR experiments were performed at the Purdue Biochemical Magnetic Resonance Laboratory, supported by National Institutes of Health grant RR-01077.

Received for publication 15 December 1979.

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THE EFFECT OF ETHIDIUM BROMIDE ON DNA INTERNAL MOTIONS

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Recent nuclear magnetic resonance studies of long DNA fragments have shown that the internal structure of B-form DNA is not rigid, but instead experiences large fluctuations in nucleotide conformation which occur with a time constant near 10^{-9} s. (Hogan and Jardetzky, 1979; Bolton and James, 1979; Klevan, et al., 1979).

Based upon ^{31}P NMR relaxation data, Klevan, et al. (1979) and Bolton and James (1979) have proposed that the backbone phosphates of DNA experience a large, fast internal motion in the helix, but that the fast phosphate motion is limited to rotations about phosphate bonds ω and ω'. As has been stated (Klevan, et al., 1979), such rotation can produce only small displacements of the two nucleosides adjoining the phosphate in the helix. Therefore, the models in Bolton and James (1979) and in Klevan, et al. (1979) do not predict fast motions which may occur at other positions in the helix.

However, as we have shown (Hogan and Jardetzky, 1979; unpublished data), the B-DNA helix must experience large fluctuations in conformation of the base and sugar as well as the