Illumination-Dependent Changes in the Intrinsic Fluorescence of Bacteriorhodopsin†

Roberto A. Bogomolni, Lisa Stubbs, and Janos K. Lanyi*

ABSTRACT: Bacteriorhodopsin fluorescence in the ultraviolet region shows a broad band around 330 nm. Actinic illumination in the visible band (4 μs flash, 575 nm) elicits cyclic fluorescence and absorbance changes in the ultraviolet. The fluorescence decreases with a half-time of about 40 μs at 22 °C and 120 μs at 12 °C closely following the absorbance change at 410 nm due to photointermediate M. The recovery time, however, is longer than the absorbance change at 410 nm and corresponds to that of the regeneration of bacteriorhodopsin. The fractional changes in fluorescence span a broad spectral range with peaks at about 330, 350, and 385 nm. Absorbance changes in the ultraviolet show an increase between 330 and 400 nm corresponding to the tail of the 410-nm peak, and a spectral shift to longer wavelength with maximum and minimum at 300 and 275–285 nm, respectively. The fluorescence emission spectrum at 1 ms after the flash (corresponding largely to the photointermediate M_{410}) was calculated and shows peaks at 304 and 328 nm and a broad shoulder at longer wavelengths, having 90%, 50%, and 35% (or less) of the original bacteriorhodopsin fluorescence intensity, respectively. Cesium chloride (1 M) selectively quenches the 350-nm fluorescence and markedly decreases the fluorescence change in that region. These results suggest that of the tryptophans in bacteriorhodopsin one or more is exposed to a polar environment (for example, the aqueous medium), and such tryptophan(s) as well as others are significantly quenched in the transition to M_{410}. The fluorescence changes may reflect changes in the spatial disposition of these residues, in their interaction with the chromophore, or in their ionization state.

Bacteriorhodopsin is a retinal–protein complex, which forms a two-dimensional crystalline array in the purple membranes of *Halobacterium halobium* (Oesterhelt & Stoeckenius, 1971; Blaurock & Stoeckenius, 1971). The apoprotein contains a single polypeptide chain of 26 000 molecular weight (Oesterhelt & Stoeckenius, 1971; Bridgen & Walker, 1976) and appears to be organized into seven α-helical segments (Henderson & Unwin, 1975), each spanning the width of the membrane. The retinal moiety is attached to one of these helical chains via a protonated Schiff-base linkage with a lysine ε-amino group (Oesterhelt & Stoeckenius, 1971; Bridgen & Walker, 1976). The exact spatial position of the retinal is not known yet, but results obtained with a variety of methods suggest that it is inclined 23° from the plane of the membrane (Bogomolni et al., 1977; Heyn & Cherry, 1977; R. W. Wilson, personal communication) and is nearer to one of the membrane surfaces (King et al., 1977).

When bacteriorhodopsin is illuminated, the pigment undergoes a rapid (10–20 ms) cyclic photochemical reaction with at least five intermediates, which have been identified by their distinct absorption bands in the visible region (for a recent review, see Lozier & Niederberger, 1977). Much of the kinetic data obtained from flash spectroscopy can be interpreted as reflecting a linear sequence of reactions involving the entities K_{590}, M_{410}, N_{520}, O_{640}, and finally BR_{570}. Recent evidence indicates, however, that the cyclic reaction may be more complicated. Associated with these reactions is the release and uptake of protons, probably during the $L_{550} \rightarrow M_{410}$ and the $O_{640} \rightarrow BR_{570}$ reactions, respectively, monitored by the appearance and disappearance of protons from the medium (Lozier et al., 1976). Resonance Raman studies have shown that the Schiff-base is deprotonated in intermediate M_{410} (Lewis et al., 1974), and recently it was found that in an M-like intermediate, stabilized in 2 M Gdn·HCl[1] at high pH, the retinal has shifted to the 13-cis configuration (Pettei et al., 1977). The release and uptake of protons are on different sides of the membrane (Lozier et al., 1976), and thus bacteriorhodopsin acts as a light-driven pump for protons, capable of replacing the respiratory chain in the creation of protonmotive force across the cytoplasmic membrane of *H. halobium* (Bogomolni et al., 1976; Belliveau & Lanyi, 1977).

Most of the information available about the light-induced events in bacteriorhodopsin is derived from changes in the chromophore absorption spectrum in the visible region, which reflect largely the behavior of the retinal and its immediate surroundings. Since the unidirectional translocation of protons must involve functional groups of the protein itself, it seems reasonable to assume that during the photochemical cycle some rearrangement of the residues takes place.

One of the properties that reports on conformational effects in proteins is their intrinsic fluorescence. Such fluorescence emission (observed between 300 and 360 nm) usually originates from tyrosine and tryptophan residues. Oesterhelt & Hess (1973) detected light-induced decreases in the fluorescence of bacteriorhodopsin in ether-saturated saline solution. In this system, where the cyclic light reaction is slowed down by three orders of magnitude, the fluorescence decrease followed the kinetics of the chromophore bleaching. The results suggested that conformational changes might occur in the apoprotein during illumination. Weak fluorescence from the chromophore in the far red region has also been reported (Lewis et al., 1976), but its relationship to the photochemical cycle has not been studied.

† From the Extraterrestrial Biology Division, Ames Research Center, National Aeronautics and Space Administration, Moffett Field, California 94035, and the Department of Biochemistry and Biophysics, and Cardiovascular Research Institute, University of California, San Francisco, California 94143. *Received August 29, 1977.* This work was partly supported by National Institutes of Health Grant No. 1RO1 GM23651-01 and NASA Grant NSG 7151.

[1] Abbreviation used: Gdn·HCl, guanidine hydrochloride.

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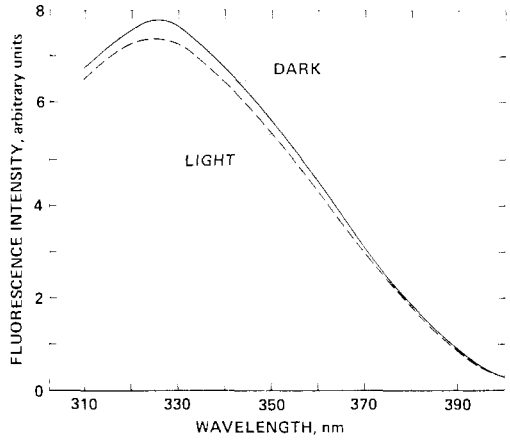

FIGURE 1: Intrinsic fluorescence (uncorrected) of bacteriorhodopsin in the dark and under actinic illumination. Purple membranes in 1 M NaCl, at pH 6.3. Fluorescence excitation at 287 nm. Details described under Materials and Methods. A Corning 7-54 UV-pass filter was used to protect the photomultiplier above 400 nm.

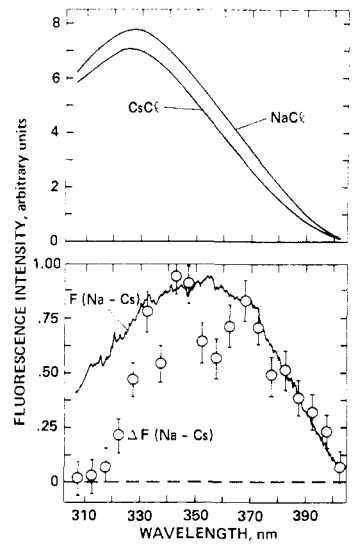

FIGURE 2: Quenching of bacteriorhodopsin fluorescence by cesium chloride. Upper graph: emission spectra, determined in 1 M NaCl or CsCl, pH 6.3; excitation wavelength 287 nm. Lower graph: the solid line is the difference between emission spectrum, determined in NaCl, and emission spectrum, determined in CsCl; open circles with error bars show the difference between dark minus light difference spectrum in NaCl and dark minus light difference spectrum in CsCl.

In the study reported here we attempted to describe the intrinsic UV fluorescence of bacteriorhodopsin in more detail, and to determine the changes during the rapid cyclic reaction following light flashes. The results suggest that several tryptophan residues are affected in the protein, among them one or more exposed to the aqueous medium. The kinetics of the fluorescence changes coincide closely with events involving the retinal residue during the deprotonation and reprotonation of the Schiff base group.

Materials and Methods

Purple membranes were isolated and purified according to Oesterhelt & Stoeckenius (1974). The spectroscopic measurements were carried out either in distilled water or in 1 M NaCl or CsCl, and the pH was adjusted with HCl. Concentration of bacteriorhodopsin was determined assuming ϵ_{570} = 63 000 M^{-1} cm^{-1} (Oesterhelt & Hess, 1973), and was 8.4 × 10^{-6} M ±10% in all experiments. Temperature, unless specified otherwise, was 22 ± 2 °C.

Steady-state fluorescence was determined in a Schoeffel Model 1000 spectrofluorimeter (right-angle geometry), modified to include a PAR Model 191 optical chopper in the exciting beam and provisions for illuminating the sample chamber with a GE EJL (150 W) lamp from the side opposite to the excitation light source. The sample was contained in a 2 × 10 mm fluorescence cuvette, with 2-mm pathlength for both the fluorescence excitation and actinic beams. Wavelengths for the actinic light were selected with Baird-Atomic interference filters (generally 575 nm). Wavelengths for excitation and emission were selected with monochromators between the light source and the sample chamber, and between the sample chamber and the detector, respectively. The detector, an EMI 9659 QA photomultiplier, was protected additionally with Corning cut-off and interference filters. The photomultiplier signal was acquired with a PAR Model JB-4 lock-in amplifier, tuned to the frequency of the optical chopper (223 cycles/s), and the output was recorded in a Nicolet 1072 signal averaging computer. When flash-induced changes in fluorescence were measured, the actinic light was either an R. Bosch Cornet 100 flash unit (flash duration 0.5 ms), or a USSI Strobrite Type 3015 stroboscope (flash duration 4 μs) and the excitation beam was not chopped. For these measurements, a Tektronix 1A7A amplifier was used.

Absorbance changes were recorded in the same instrument but with different geometry: a Hitachi deuterium lamp or a 50-W Tungsten lamp was used as the measuring beam light source, placed in a straight line from the detector. In this configuration monochromators on both sides of the sample chamber were used to isolate the wavelength of interest.

Special resolution was 13.2 nm (4-mm slits), unless otherwise stated. The time-constant for steady-state measurements was 0.1 s, and as low as 20 μs for measurements of the kinetics, the latter limited largely by the speed of the digitizer in the signal averager (9 bits, nominally 10 μs/point). Absorbance changes were calculated according to Lozier et al. (1976).

Since some bleaching took place in bacteriorhodopsin during exposure to UV radiation, care was taken to change the samples often enough to avoid appreciable alterations in optical properties.

Results

Steady-State Fluorescence of Bacteriorhodopsin. The intrinsic fluorescence spectrum of bacteriorhodopsin is relatively featureless (Figure 1), as in many other proteins (Kronman, 1976). The band maximum at 330 nm indicates that the fluorescence is dominated by tryptophans, and a slight shoulder at 350 nm suggests the presence of tryptophan exposed to an aqueous environment. Illumination at an approximate light intensity of 1 × 10^6 ergs s^{-1} cm^{-2} between 520 and 700 nm causes a fluorescence decrease of about 4% (Figure 1). The relationship of this fluorescence quenching to the amount of bacteriorhodopsin bleached will be discussed below in connection with the calculation of the fluorescence emission spectrum of the phototransient at 1 ms. Qualitatively, the result is similar to the effect reported earlier by Oesterhelt & Hess (1973).

Heavy metal ions, such as Cs^+, are effective quenchers of tryptophan fluorescence when the amino acid is located at a site accessible to water (Chen, 1976). The quenching is collisional and results in no band shift. Cesium chloride, at a 1 M concentration, reduces the fluorescence of bacteriorhodopsin relative to that in NaCl (Figure 2), and the NaCl minus CsCl difference spectrum contains primarily emission at 350 nm,

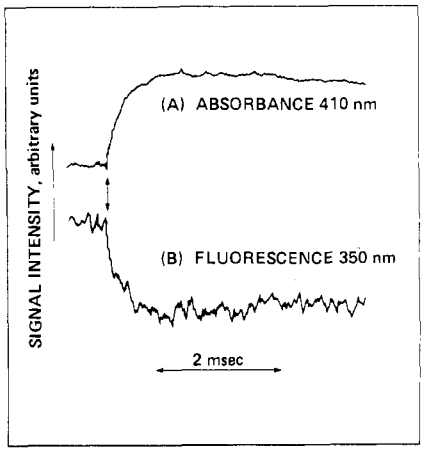

FIGURE 3: Rise kinetics of light-induced fluorescence and absorbance changes in bacteriorhodopsin. Actinic illumination was a 4-μs flash, at 1 ms after the beginning of the traces as indicated by arrow, while absorbance at 410 nm (trace A) and fluorescence at 350 nm, excitation 285 nm (trace B) was followed. Details of the measurement described under Materials and Methods. Signal averaging was continued for 128 flashes for trace A and 4096 flashes for trace B. The magnitudes of the changes were adjusted to be approximately equal. Purple membranes in distilled water, pH 6.3. Temperature reduced to 12 °C in order to slow down the reaction.

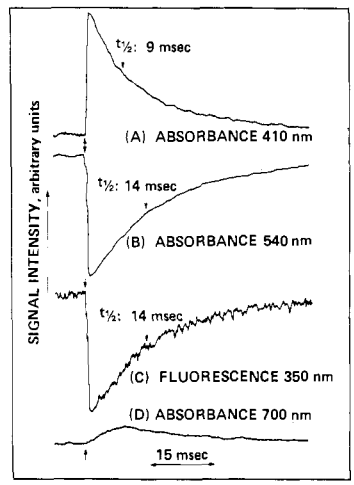

FIGURE 4: Decay kinetics of light-induced fluorescence and absorbance changes in bacteriorhodopsin. Conditions similar to those in Figure 3, but the pH was 5.0 and the temperature 18 °C. Trace: A: absorbance at 410 nm (corresponding to the M_{410} intermediate). Trace B: absorbance at 540 nm (corresponding to BR). Trace C: fluorescence at 350 nm, with excitation at 285 nm. Trace D: absorbance at 700 nm (corresponding to the O_{640} intermediate). The magnitudes of the upper three traces were adjusted to be approximately the same. Arrows indicate time for half-maximal decay ($t_{1/2}$).

as expected. Illumination causes a decrease of fluorescence in CsCl solution also, but somewhat less than in NaCl. The difference between the effect of actinic light in the two salt solutions approximately equals the CsCl-quenched portion of the emission band (Figure 2, lower graph), indicating that part of the fluorescence changes originate from exposed tryptophan group(s).

Kinetics of Fluorescence Changes. The formation and decay of the various photointermediates in bacteriorhodopsin can be followed by flash spectroscopy of light absorption in the visible region. Figure 3 shows the kinetics of formation for the M_{410} intermediate, its rate slowed down sufficiently at 12 °C for observation with the time-resolution available. Also shown in this graph is a trace of fluorescence intensity, measured under the same conditions, with the same sample. The formation of M_{410} and of the species with lowered fluorescence can both be characterized with a half-rise time of 120 μs. Since the intermediate previous to M_{410} arises with a time-constant of <5 μs, and the intermediate after M_{410} with a time-constant of several ms, the observed lowered fluorescence is tentatively assigned, on solely kinetic grounds, to M_{410}.

Transient accumulation of the photointermediate O_{640} is enhanced at lower pH (e.g., below pH 6) (Lozier & Niederberger, 1977). At high pH the rates for the return of the fluorescence and for the decay of absorbance at 410 nm coincide closely (not shown). At pH 5 BR is formed more slowly than the decay of M_{410}, however (Figure 4), and the transient accumulation of O_{640} can be observed at long wavelengths. At pH 5 the decay of the fluorescence change is slower than the decay of M_{410} (Figure 4) but coincides with the formation of BR. From these traces it is tentatively concluded that the lowered fluorescence, relative to BR, is a property of both M_{410} and O_{640}.

Light-Dependent Absorption Changes in the Ultraviolet. Partly to avoid artifacts in the fluorescence measurements, due to transmissivity changes in the purple membrane suspensions, and partly because absorption changes for aromatic amino acids also reflect changes in the proteins (Wetlaufer, 1962), the light-induced absorption changes of bacteriorhodopsin in the ultraviolet were followed. Although detailed kinetic studies

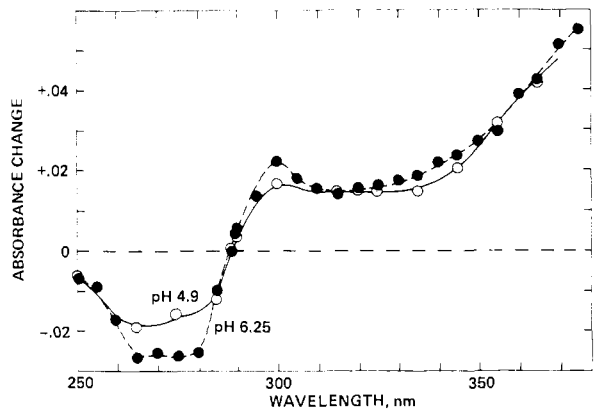

FIGURE 5: Light-induced ultraviolet absorbance changes in bacteriorhodopsin. The absorption changes were measured at 1 ms after actinic flashes of 0.5-ms duration and with 10-mm pathlength. Generally 4–8 flashes were averaged. Symbols: (⊙) pH 4.9; (●) pH 6.25. Spectral resolution 6.6 nm (2-mm slits) at pH 6.25, and 13.2 nm (4-mm slits) at pH 4.9.

were not made, the absorption changes in general followed the pattern of fluorescence changes in Figure 4. It was assumed that the absorption properties measured 1 ms after the light-flash would reflect largely those of the intermediate M_{410}.

The amplitude of absorption changes at 1 ms is plotted against wavelength in Figure 5 at pH 4.9 and 6.25. The long wavelength region represents increase in absorbance due to the short wavelength end of the chromophore absorption band at 410 nm. Below this region absorption decrease at 265 to 280 nm and increase at 300 nm are seen. The extinction of tyrosine between 275 and 285 nm decreases with loss of H^+, and increases at 295 nm (Wetlaufer, 1962). Similarly, deprotonation of tryptophan results in absorption increase at 300 nm (Donovan et al., 1961). The results obtained with bacteriorhodopsin (Figure 5) are consistent with the deprotonation of tyrosine or tryptophan in the BR to M_{410} transition.

The magnitude of the absorption changes are too small, by a factor of 5–6, to cause attenuation of the emitted fluorescence which would account for the observed changes in emission

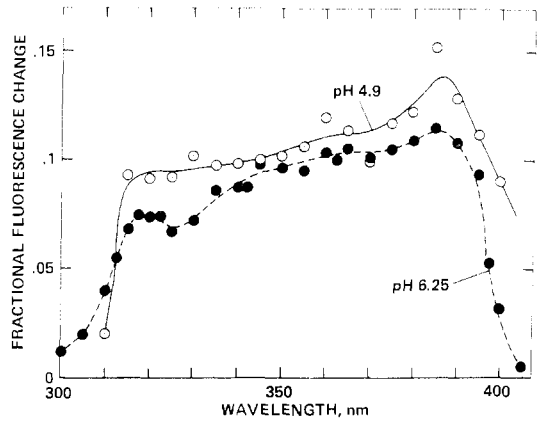

FIGURE 6: Light-induced changes in the fluorescence emission spectrum of bacteriorhodopsin. The fractional change in fluorescence intensity ($\Delta F/F$), at 1 ms after flash, is plotted against emission wavelength. Conditions as in Figure 5. The fraction of bacteriorhodopsin photoactivated by the flash is about 0.16. Spectral resolution 6.6 nm (2-mm slits).

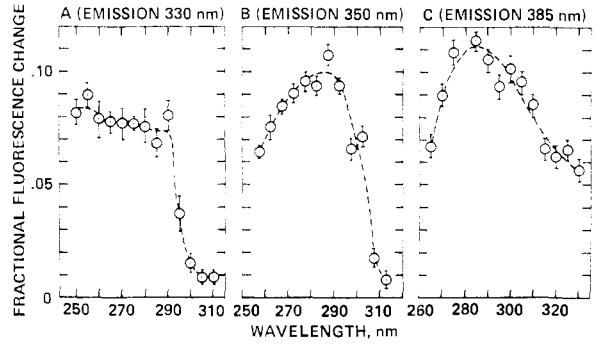

FIGURE 7: Excitation spectra for the light-induced fluorescence changes in bacteriorhodopsin. Conditions and procedure similar to those in Figure 6, but the measurements were only done at pH 6.3. (A) Emission at 330 nm; (B) emission at 350 nm; (C) emission at 385 nm.

intensity. To minimize such effects on the excitation light, the excitation wavelength was generally set near the isosbestic point of 288 nm. Furthermore, the fluorescence changes are kinetically separable from the absorption change at 410 nm (Figure 4), and the spectrum of the fractional changes in fluorescence (see below) does not correspond to the absorption changes in Figure 5. Because of these considerations it is unlikely that the observed fluorescence intensity changes are caused by transmittance changes in the sample.

Excitation and Emission Spectra for the Fractional Fluorescence Decrease at 1 Millisecond. The relative magnitude of the fluorescence change, $\Delta F/F$, was determined at different wavelengths. The amplitude of the fractional change at 1 ms after the light flash is plotted against emission wavelength in Figure 6. The spectra at pH 4.9 and 6.25 are similar, and indicate that at least three kinds of emitting species are involved: at 330, 350, and 385 nm. No significant differences in decay kinetics were observed at the three wavelengths. Excitation spectra for each of these emission bands were obtained, and are shown in Figure 7. The excitation spectrum for 330-nm emission (Figure 7A) resembles a typical protein excitation spectrum. The excitation spectra for longer emission wavelengths (Figures 7B and C) tend to shift toward longer wavelengths, showing selection for those fluorescent species which absorb at longer wavelengths. This effect is similar to those reported for other proteins, where tryptophans emitting from different environments can be preferentially excited at different wavelengths (Kronman, 1976). Although the nature of the species which are excited at the various wavelengths is not clear, the differences in excitation spectra generally confirm the existence of distinct fluorescent species, suggested by the emission spectrum (Figure 6).

Discussion

Bacteriorhodopsin has been reported to contain four tryptophans and 10–11 tyrosine residues (Oesterhelt & Stoeckenius, 1971; Bridgen & Walker, 1976), although recently seven tryptophans were reported (Keefer & Bradshaw, 1977). As expected, the fluorescence emission bands from individual residues overlap extensively and cannot be resolved. By selective quenching with CsCl, it has been possible to separate the spectral contribution of one kind of residue, tryptophan exposed to an aqueous environment. The general position of the emission band near 330 nm (Figure 1) indicates that tyrosines which emit at 305 nm contribute only a minor fraction of the UV fluorescence, in spite of their larger abundance in the protein.

Light-dependent decrease in fluorescence occurs in bacteriorhodopsin for three kinds of residues, emitting at 330, 350, and 385 nm. The first two should correspond to tryptophans in hydrophobic and aqueous environments, respectively (Kronman, 1976). The third kind of residue is unidentified at this time, but this emission is also reversibly modulated by visible actinic light, which makes it unlikely that it is due to an impurity. Various effects could cause a shift in emission of aromatic amino acids toward longer wavelengths. A shift to the red is observed upon long wavelength band edge excitation of tryptophan in aqueous solution (Tatischeff & Klein, 1975). This shift originates from strong solute–solvent interactions and requires slow solvent relaxation (Itoh & Azumi, 1975), a condition incompatible at room temperatures with an aqueous environment. However, the environment of the indol and aromatic residues in a protein is not necessarily "aqueous." Alternatively, a red shift could result from direct interaction of tryptophan with the retinal chromophore or from ionization (Stryer, 1966) of the residue.

A possible photoproduct absorbing at the long wavelength band edge of the main emitter and fluorescing at 380 nm should also be considered. To account for the modulation of the emission of actinic light, such a product must still be a component of, or alternatively strongly interact with, the chromoprotein. Because we did not detect a time dependency in the intensity of the long wavelength fluorescence changes, the formation of a cumulative photoproduct during the measurement is not a plausible explanation. However, such species may exist naturally in bacteriorhodopsin which is normally irradiated under physiological conditions. Appearance of an emission band at around 380 nm has been observed in aqueous tryptophan soutions at room temperature exposed to solar radiation (Tatischeff et al., 1976), tentatively assigned to a C_3-hydroxylated derivative. Tryptophan residues in bacteriorhodopsin may occur naturally in such modified form.

Little or no change in fluorescence occurs at wavelengths below 310 nm, where tyrosine emits, but fluorescence is extensively quenched at longer wavelengths. Thus, as shown in Figure 8, the fluorescence emission spectrum, which is tentatively assigned to the M_{410} intermediate, and calculated from fluorescence and absorption changes observed at 1 ms after light-flashes (see legend), shows distinct peaks at 304 nm and 328 nm and appears more structured. The extent of quenching is about 10% near 300 nm, 50% at 328 nm, and 65% or more at longer wavelengths.

It seems reasonable to assume that the light-initiated events

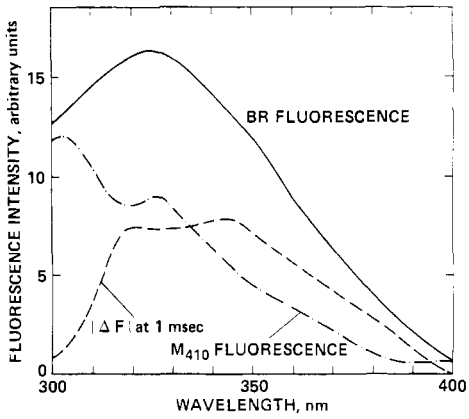

FIGURE 8: Fluorescence emission spectrum of the M_{410} intermediate. The fractional fluorescence changes at 1 ms in Figure 6 (at pH 6.25) were converted to absolute change (- - -) and subtracted from the fluorescence spectrum of the fraction (0.16) of bacteriorhodopsin undergoing cycling in each flash (—). The difference (- · · · -) gives the fluorescence emission spectrum of the emitting species at 1 ms, which should correspond largely to M_{410}.

in bacteriorhodopsin are not restricted to the immediate neighborhood of retinal and therefore might not be detected by changes in optical properties in the visible region. We had expected that such events could be accessible by measuring the fluorescence of aromatic amino acid residues. This does not appear to be the case: the major part, if not all, of the fluorescence changes can be correlated kinetically with the formation of the M_{410} and O_{640} intermediates (Figures 3 and 4). If other events occur, with different time constants, they must be restricted to regions not containing tryptophans or tyrosines sensitive to their environment. The quality of the data has not allowed the resolution of any differences between the fluorescence of M_{410} and O_{640}. Thus, from the point of view of fluorescence properties, the photochemical cycle can be described by the following scheme: BR → (M_{410}, O_{640}) → BR.

To the extent that the emission of tryptophans reflects the conformational properties of bacteriorhodopsin, it may be concluded that light-dependent changes in the disposition of amino acid residues do occur during the second half of the photochemical cycle. Alternately, or additionally, the fluorescence decreases may reflect tryptophan–tyrosine interaction which would be sensitive to the ionized state of tyrosine. Deprotonation of tyrosine would result in an absorption difference spectrum similar to the one reported in Figure 5. Studies with peptides containing both tryptophan and tyrosine had shown that ionization of the phenol group causes quenching of tryptophan fluorescence, possibly due to energy transfer from the indol to the phenolate ion (Cowgill, 1963) because the absorption band of ionized tyrosine overlaps the emission band of tryptophan. A third possibility is that the fluorescence changes originate from increased tryptophan–retinal resonance energy exchange. Evidence for the existence of such exchange in a borohydride-reduced photoproduct of bacteriorhodopsin was recently given by Schreckenbach & Oesterhelt (1977).

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