EVIDENCE FOR RHODOPSIN REFOLDING DURING THE DECAY OF META II

KENNETH J. ROTHSCHILD,* JOHN GILLESPIE,* AND WILLEM J. DEGRIP‡
*Departments of Physics and Physiology, Program in Cellular Biophysics, Boston University, Boston, Massachusetts 02215; and ‡Department of Biochemistry, University of Nijmegen, Nijmegen, The Netherlands

ABSTRACT Fourier transform infrared difference spectroscopy (FTIR) reveals that the Meta II intermediate of the rhodopsin bleaching cascade is structurally distorted relative to rhodopsin. In addition to previously detected alterations in the state of carboxyl groups, a small part of the protein back-bone undergoes a conversion from α-helical to β-type structure. All of these changes partially reverse during Meta II decay. This evidence together with FTIR studies of earlier photointermediates indicates that of the known photointermediates the protein structure of Meta II is the most distorted. It is concluded that light causes rhodopsin to convert into a conformationally distorted form (Meta II), which subsequently refolds into a more rhodopsin-like conformation (opsin).

INTRODUCTION

The primary process in visual transduction involves light absorption by rhodopsin, an integral protein found in the photoreceptor membrane of vertebrate rod cells. Rhodopsin is formed by covalent interaction of the apoprotein, opsin, with a small chromophoric group, 11-*cis* retinal (1). Light absorption causes rapid isomerization of the 11-*cis* configuration of retinal to the all-*trans* form, which is thought to induce conformational changes in the protein part of the complex (2). This generates the well known bleaching cascade, a series of spectroscopically distinct intermediates (Fig. 1).

A key step for visual transduction is the formation of Meta II, which binds and activates a specific G-protein (transducin). This interaction initiates a signal-conveying and amplifying system in the rod outer segment resulting in activation of a cyclic-GMP phosphodiesterase (3, 4). The interaction of Meta II with the G-protein can be blocked via phosphorylation of Meta II, a reaction catalyzed by a specific protein kinase (4) or by thermal decay of Meta II. The latter process has a half time of ~1 min at 37°C (5) and is the first of the two slower steps that results in the release of all-*trans* retinal and formation of opsin (5–7).

Since rhodopsin bleaching and regeneration is a cyclic process, the protein conformational changes induced by bleaching must eventually be reversed. This report addresses in part the question of when this refolding occurs. One can imagine two distinct possibilities. (*a*) The protein changes that have occurred by Meta II might reverse upon rebinding of 11-*cis* retinal to opsin. (*b*) Conformational refolding might occur during the Meta II decay, which involves formation of the long lived intermediate Meta III as well as opsin and all-*trans* retinal (5–7).

Indirect biochemical evidence supports but does not prove alternative *b* (6). For instance, like rhodopsin, both opsin and Meta III do not activate the G-protein and are much poorer substrates for the protein kinase than Meta II (4). Further, the chemical reactivity of opsin and Meta III is more comparable to rhodopsin than to Meta II (6). Finally, the steric requirements of the chromophore binding pocket (8) and the kinetic parameters of the binding reaction of 11-*cis* retinal with opsin (5) are also suggestive of conformational similarity between opsin and rhodopsin. However, so far no direct structural evidence has been reported to show that the decay of Meta II is indeed accompanied by conformational refolding.

To address this question, we have utilized Fourier transform infrared (FTIR) difference spectroscopy, which has been applied previously to study conformational changes up to the Meta II stage of bleaching (9–12).

METHODS

FTIR measurements were made as described before (9, 10) on fully hydrated multilamellar films of photoreceptor membrane formed using the isopotential spin-dry method (13). These films have been shown to undergo normal bleaching and are fully regenerable with 11-*cis* retinal (14).

All spectra were recorded at 20°C, using a spectrometer (model 60 SX; Nicolet Analytical Instruments, Madison, WI) equipped with a Deuterated Triglycine Sulfate detector. A 100-W fiber optic illuminator (Dolan-

Correspondence should be addressed to Kenneth J. Rothschild, Department of Physics, Boston University, Boston, MA 02215.

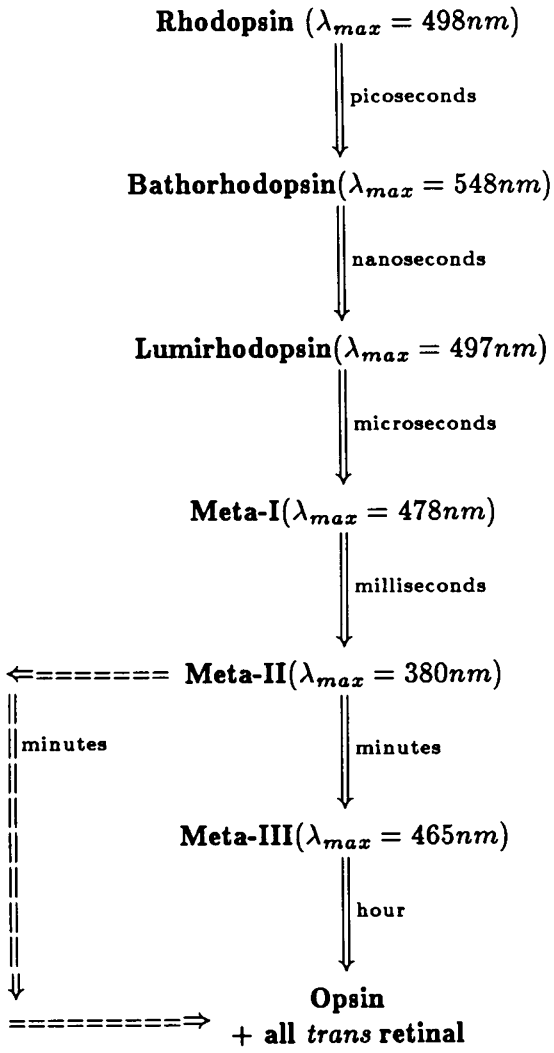

FIGURE 1 Bovine rhodopsin bleaching sequence at room temperature with absorbance maxima for different intermediates along with their approximate decay times.

Jenner Industries, Inc., Woburn, MA) filtered with a 500-nm long-band pass filter was used for bleaching.

An important aspect of the FTIR measurements was the ability to obtain high signal-to-noise difference spectra in approximately 1-min periods. This was accomplished by recording each spectrum at 8 cm^{-1} resolution, a spectrum consisting of 300 scans. The mirror velocity was 0.4 cm/s, with 2,048 data points in the interferogram. The Meta II decay difference spectra were computed by subtracting a spectrum recorded 1.5 min after the bleaching light was turned off from successive spectra recorded at 1.5-min intervals. Under these conditions, difference spectra that compare favorably with previously reported FTIR differences (9) were obtained in 1 min rather than the normal 15 min necessary for data acquisition.

RESULTS AND DISCUSSION

In this report, we restrict ourselves to the range 1,400–1,800 cm^{-1}, where important structural groups in rhodopsin absorb. The rhodopsin-to-Meta II difference spectrum is shown in Fig. 2 (*dashed line*) and agrees with earlier reports (9, 10) except for a much improved signal-to-noise ratio. We have found that many of the IR spectral differences that appear by the Meta II intermediate decay at 20°C with a half time of ~7 min. This is most apparent from a comparison of the initial rhodopsin-to-Meta II difference spectrum with the Meta II decay spectra (Fig. 2). A comparison of the difference spectra between rhodopsin and the bleached sample after 1 min and 15 min (Fig. 3) also reveals that many of the spectral features originally present have decayed.

In general, difference spectra of photoreceptor membrane can contain contributions from the chromophore, protein, and lipid components of the membrane. Lipid exchange studies so far indicate that lipids do not significantly contribute to the changes described below (unpublished observations). For the chromophore, prominent retinal vibrations are observed in the resonance Raman spectrum of rhodopsin at 1,655 cm^{-1} (protonated C=N stretch) and 1,545 cm^{-1} (C=C stretch) (15, 16). These peaks would be expected to appear as negative features in the rhodopsin-to-Meta II transition difference spectrum. Strong peaks in these regions are observed but at higher frequencies (Fig. 3, *solid line*). Notice, however, that the rhodopsin bleaching difference spectrum (Fig. 3, *dashed line*) at 15 min after bleaching shows reduced negative peaks exactly at these frequencies. This can be explained by the presence of negative peaks in the early bleaching difference spectrum (rhodopsin to Meta II), which have partially disappeared by 15 min. This decay is observed directly in the decay difference spectra (Fig. 2, *solid lines*) and reveals large negative peaks at 1,661 and 1,557 cm^{-1} that are accompanied by positive peaks at 1,643 and 1,531 cm^{-1}. These peaks reflect structural alterations that have occurred between rhodopsin and Meta II, and have then reversed during the decay of Meta II.

As discussed below, many of the spectral changes that occur during the decay of Meta II appear to involve alterations in protein groups. (*a*) The above mentioned increases during Meta II decay at 1,557 and 1,661 cm^{-1} are accompanied by decreases of intensity at 1,643, 1,684, and 1,531 cm^{-1}. Such frequency shifts are characteristic of rearrangement of the peptide backbone and are consistent with a shift from a β-sheet or turn to an α-helical structure (17, 18), reversing the alterations that occur during the formation of Meta II. This rearrangement could involve at most a few peptide groups since the changes in absorbance (~0.01) are small relative to the absolute amide I absorbance (~1.0). We also note that an amide I frequency as high as 1,660 cm^{-1} may also be indicative of an α_{II}-helix (19) and is close to the amide I frequency observed in bacteriorhodopsin (20). (*b*) The rhodopsin-to-Meta II difference spectra are very similar in this region, irrespective of whether they are taken in D_2O or H_2O (9, 11). Hence, it is unlikely that the water band at 1,640 cm^{-1} contributes to the difference spectrum. (*c*) Additional peaks observed in the decay spectra include the peaks above 1,700 cm^{-1} assigned to carboxyl groups (10) and

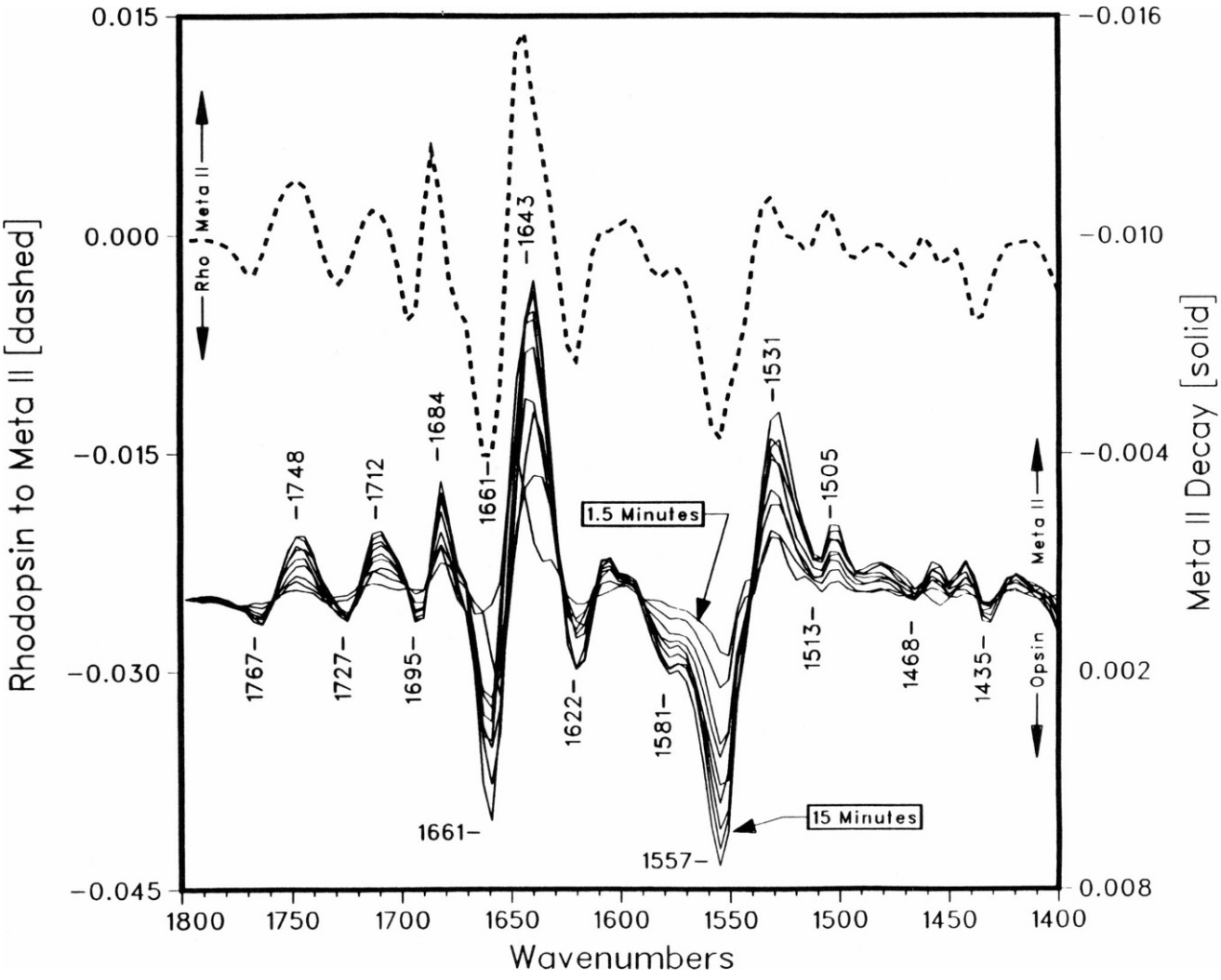

FIGURE 2 Protein conformational changes from rhodopsin-to-Meta II are partially reversed during Meta II to opsin decay. FTIR difference spectra in the 1,800–1400 cm^{-1} region for rhodopsin-to-Meta II transition (*dashed line*) and Meta II decay (*solid lines*). Note that the Meta II decay spectra have been flipped for comparison with the rhodopsin to Meta II difference spectrum.

positive peaks at 1,515 and 1,264 cm^{-1} characteristic of tyrosine along with negative peaks at 1,505 and 1,273 cm^{-1} characteristic of tyrosinate (21). All of these groups undergo an alteration between rhodopsin and Meta II that reverses during the Meta II decay.

To check whether the above mentioned reversals could be due to the unlikely possibility that rhodopsin regeneration might occur due to formation of 11-*cis* retinal from free all-*trans* retinal during illumination, we studied bleaching in the presence of hydroxylamine, which reacts rapidly with Meta II and Meta III, as well as free retinal, to form retinaloxime. The rhodopsin-to-Meta II difference spectrum after 1 min (cf., Fig. 4) exhibits a marked reduction in many of the peaks associated with protein changes, particularly near 1,665, 1,642, 1,554, and 1,532 cm^{-1}. This spectrum closely resembles the difference spectrum (Fig. 3, *dashed lines*) between rhodopsin and the Meta II decay products 15 min after bleaching without hydroxylamine. In addition, only small changes in the rhodopsin to Meta II difference spectrum with hydroxylamine were observed over a period of 15 min. Hence, it appears that the presence of hydroxylamine either significantly speeds up the protein refolding or that it completely blocks the protein changes that normally occur up to Meta II formation. The first explanation must be correct since hydroxylamine does not inhibit Meta II formation but is known to rapidly abolish Meta II by conversion into opsin and retinaloxime, as well as to preclude formation of Meta III (22).

Fig. 5 shows the time course for the decay of several protein related peaks after bleaching at 20°C. The half time of all peaks is ~7 min at 20°C and the decay rate is enhanced by increasing temperature ($t_{1/2}$ = 3 min at 30°C; not shown). This compares well with the decay of Meta II as measured in aqueous suspensions of photoreceptor membrane (5, 22). It is found that there is 50–60% return

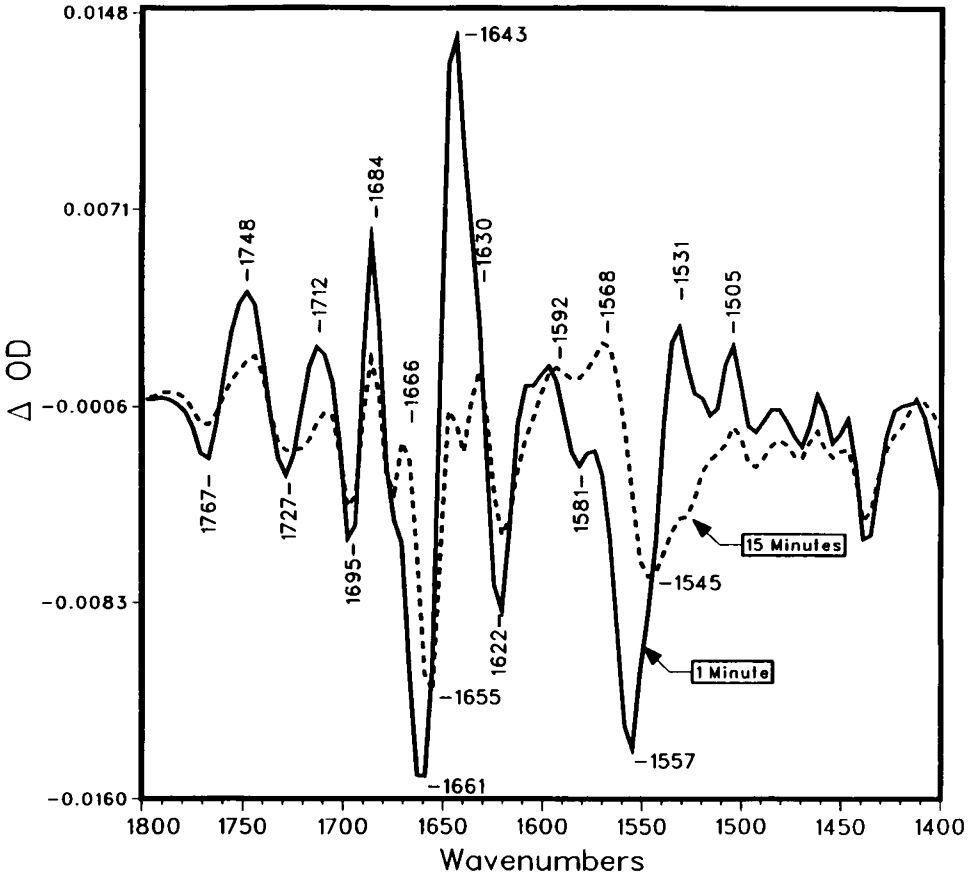

FIGURE 3 Rhodopsin bleaching difference spectrum at 1 min and 15 min. Difference spectra were computed by taking the difference between a spectrum recorded immediately before a 1-min light exposure and spectra that were recorded starting at 30 s (*solid line*) and 15 min (*dashed line*) after light exposure. The acquisition time of each spectrum was 1 min.

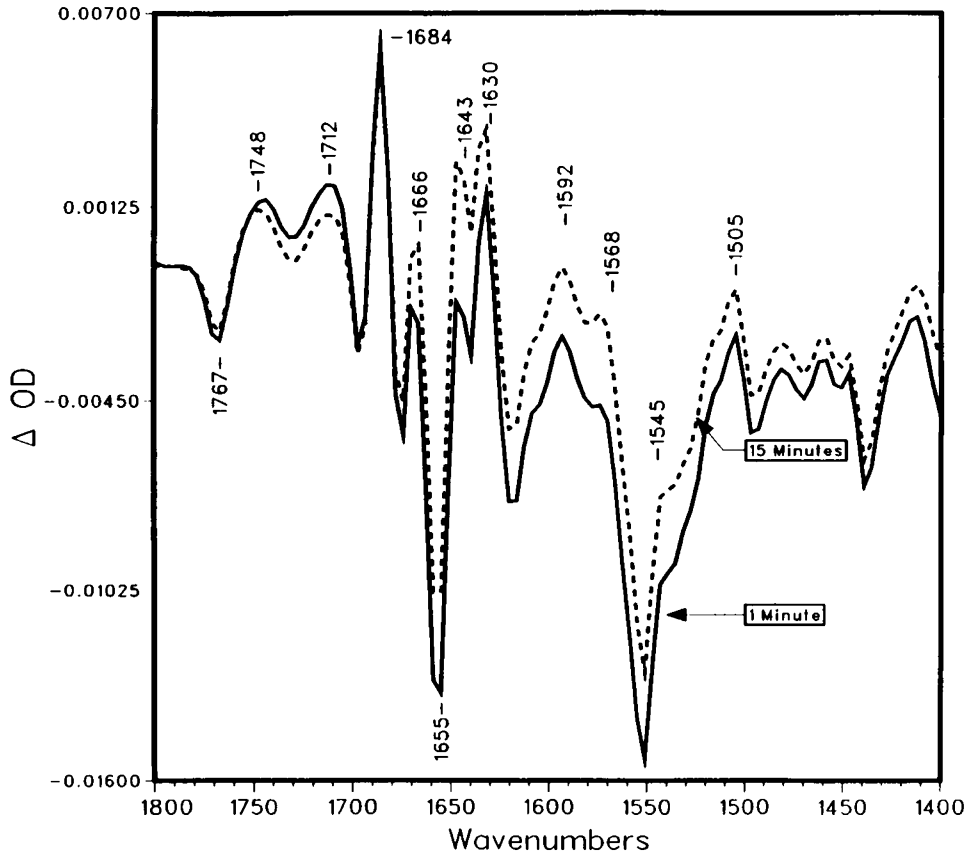

FIGURE 4 Rhodopsin bleached in the presence of 0.5 M hydroxylamine. Difference spectra were recorded as described in Fig. 3 caption.

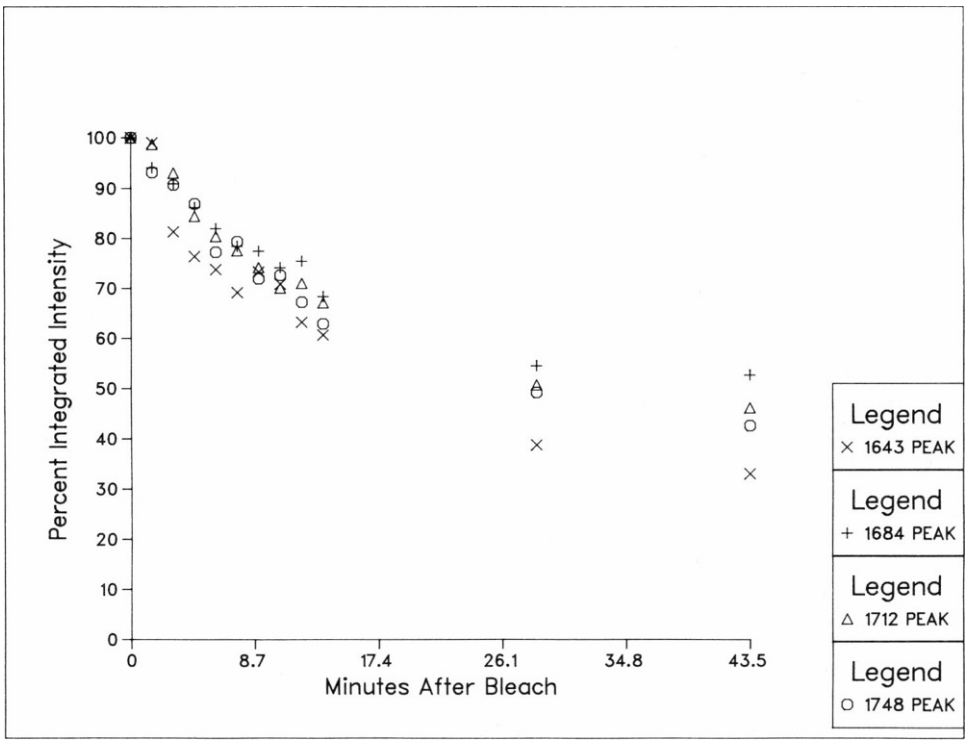

FIGURE 5 A plot of the absolute integrated intensity of selected peaks in the Meta II decay difference spectrum at 20°C (shown in Fig. 2) as a function of time after the bleaching light is turned-off.

of the protein to its original rhodopsin conformation after 15 min. A slower decay was also observed beyond 15 min, although complete refolding was not observed even after 3 h.

Although Meta II decays to a mixture of 60–70% Meta III and 30–40% opsin (22), the coexistence of these two species cannot explain the observed incomplete refolding. In particular, the 15-min Meta II decay difference spectrum is very similar to the 1.5-min difference spectrum in the presence of hydroxylamine (Fig. 4), which rapidly converts all photoproducts into opsin and retinaloxime. Hence, Meta III and opsin probably have very similar conformations, in agreement with less direct biochemical evidence (6, 22). It is conceivable that the absence of the chromophore allows the interior of the protein to adopt a dynamic equilibrium between two states. One state would resemble the rhodopsin configuration. The other state would be a Meta II-like conformation, however without expression of the specific Meta II signal-sites at the exterior of the protein. Alternatively, the residual Meta II-like features may reflect a subpopulation of the original Meta II that has not completely undergone refolding. In this regard, it will be important to determine whether the extent of refolding is temperature and pH dependent, as is found in the case of the the Meta I-to-Meta II equilibrium (6). It is unlikely, however, that the incomplete refolding reflects a homogeneous population of bleaching intermediates that have partially returned to a rhodopsin conformation since many of these peaks are assigned to single residues (10), and the spectral changes observed during the decay of Meta II have similar frequencies to the changes produced during the formation of Meta II from rhodopsin.

CONCLUSIONS

In conclusion, we provide the first direct evidence that the decay of Meta II involves refolding of the protein back to a more rhodopsin-like conformation. There are several points to consider in regard to these findings. (*a*) Meta II appears to be the most distorted photointermediate in the photocascade. In addition to the refolding of the protein observed during Meta II decay, all other photointermediates before Meta II including lumirhodopsin have now been studied by FTIR difference spectroscopy (23) and found to exhibit less protein alteration. In particular, vibrations associated with peptide backbone alterations and carboxyl groups are found to exhibit maximum change at the Meta II state. In view of the fact that rhodopsin stores 60% of the absorbed photon energy during the initial bleaching step (9, 10, 24), it is likely that the energy driving these protein rearrangements at Meta II is directly derived from the photon energy, although the present experiments do not conclusively establish this. (*b*) The similarity between rhodopsin and opsin conformation may be related to the fact that opsin is physiologically inactive as an initiator for visual transduction. Meta II, the conformationally most distorted protein state, has a high affinity for the G-binding protein

(transducin) and the ATP-dependent protein kinase (4), but both rhodopsin and opsin show no or only low affinity for these proteins. The protein refolding we observe during Meta II decay probably reflects to some extent the conformational changes underlying this change in affinity. (*c*) The similarity between opsin and rhodopsin explains why 11-*cis* retinal binds spontaneously to opsin without any intermediate conformational steps (6). In particular, the refolding we observe during Meta II decay may restore rhodopsin's binding pocket, allowing direct access of 11-*cis* retinal but not all-*trans* or 13-*cis* retinal. Protonation of the Schiff base would not require further protein alteration. This is in agreement with recent evidence indicating that binding is determined mainly by steric constraints (8).

This research was supported by grant EY 05499 from the National Eye Institute-National Institutes of Health to K. J. Rothschild, by the Dutch organization for the advancement of basic research (ZWO-SON) to W. J. DeGrip, and a joint NATO grant (0381/83) to K. J. Rothschild and W. J. DeGrip. The acquisition of the Nicolet 60SX spectrometer was sponsored by a grant from the Instrumentation Division of the National Science Foundation. We thank P. Ahl, P. Roepe, D. Reny, G. Cordrey, D. Gray, T. Earnest, M. Tran, H. Marrero, M. Braiman, and P. Bovee-Geurts for helpful discussions and technical assistance.

Received for publication 9 June 1986 and in final form 21 October 1986.

REFERENCES

1. Wald, G. 1968. The molecular basis of visual excitation. *Nature. (Lond.).* 219:800–807.
2. Hargrave, P. A. 1982. Rhodopsin chemistry, structure and topography. *Prog. Ret. Res.* 1:1–51.
3. Stryer, L. 1985. Molecular design of an amplification cascade in vision. *Biopolymers.* 24:29–47.
4. Kühn, H. 1984. Interactions between photoexcited rhodopsin and light-activated enzymes in rods. *Prog. Ret. Res.* 3:123–156.
5. Ostroy, S. E. 1977. Rhodopsin and the visual process. *Biochim. Biophys. Acta.* 463:91–125.
6. Daemen, F. J. M., and W. J. DeGrip. 1984. Photoreceptor membranes: model for excitable membranes. *NATO Adv. Study Inst. Ser. Ser. A Life Sci* 76:225–248.
7. Matthews, R. S., R. Hubbard, P. K. Brown, and G. Wald. 1963. Tautomeric forms of metarhodopsin. *J. Gen. Physiol.* 47:215–240.
8. Liu, R. S. H., A. E. Asato, M. Denny, and D. Mead. 1984. The nature of restrictions in the binding site of rhodopsin. A model study. *J. Am. Chem. Soc.* 106:8298–8300.
9. Rothschild, K. J., W. A. Cantore, and H. Marrero. 1983. Fourier transform infrared difference spectra of intermediates in rhodopsin bleaching. *Science (Wash. DC).* 219:1333–1335.
10. DeGrip, W. J., J. Gillespie, and K. J. Rothschild. 1985. Carboxyl group involvement in the meta I and meta II stages in rhodopsin bleaching. A Fourier transform infrared spectroscopic study. *Biochim. Biophys. Acta.* 809:97–106.
11. Siebert, F., W. Mäntele, and K. Gerwert. 1983. Fourier-transform infrared spectroscopy applied to rhodopsin, the problem of the protonation state of the retinylidene Schiff base re-investigated. *Eur. J. Biochem.* 136:119–127.
12. Bagley, K. A., V. Balogh-Nair, A. A. Croteau, G. Dollinger, T. G. Ebrey, L. Eisenstein, M. K. Hong, K. Nakanishi, and J. Vittitow. 1985. Fourier-transform infrared difference spectroscopy of rhodopsin and its photoproducts at low temperature. *Biochemistry.* 24:6055–6071.
13. Clark, N. A., K. J. Rothschild, B. A. Simon, and D. A. Luippold. 1980. Surface induced orientation of multilayer membrane arrays: theoretical analysis and a new method with application to purple membrane fragments. *Biophys. J.* 31:65–96.
14. Rothschild, K. J., N. A. Clark, and K. M. Rosen. 1980. Incorporation of photoreceptor membrane into a multilamellar film. *Biophys. J.* 31:45–52.
15. Mathies, R., A. R. Oseroff, and L. Stryer. 1976. Rapid-flow resonance Raman spectroscopy of photolabile molecules: Rhodopsin and isorhodopsin. *Proc. Natl. Acad. Sci. USA.* 73:1–5.
16. Doukas, A. G., B. Aton, R. H. Callender, and T. G. Ebrey. 1978. Resonance Raman studies of bovine metarhodopsin I and metarhodopsin II. *Biochemistry.* 17:2430–2435.
17. Parker, F. S. 1983. Applications of Infared, Raman and Resonance Raman Spectroscopy in Biochemistry. Plenum Publishing Corp., New York. 83–149.
18. Krimm, S. 1983. Vibrational analysis of conformation in peptides, polypeptides and proteins. *Biopolymers.* 22:217–225.
19. Krimm, S., and A. M. Dwivedi. 1982. Infrared spectrum of the purple membrane: clue to a proton conduction mechanism? *Science (Wash. DC).* 216:407–408.
20. Rothschild, K. J., and N. A. Clark. 1979. Anomalous amide I infrared absorption of purple membrane. *Science (Wash. DC).* 204:311–312.
21. Rothschild, K. J., P. Roepe, P. L. Ahl, T. N. Earnest, R. A. Bogolmolni, S. K. Das Gupta, C. M. Mulliken, and J. Herzfeld. 1986. Evidence for a tyrosine protonation change during the primary phototransition of bacteriorhodopsin at low temperature. *Proc. Natl. Acad. Sci. USA.* 83:347–351.
22. Van Breugel, P. J. G. M., P. H. M. Geurts, S. L. Bonting, and F. J. M. Daemen. 1979. Biochemical aspects of the visual process XL: spectral and chemical analysis of metarhodopsin III in photoreceptor membrane suspensions. *Biochim. Biophys. Acta.* 557:188–198.
23. Rothschild, K. J., and W. J. DeGrip. 1987. FTIR studies of the rhodopsin transduction mechanism. *Photobiochem. Photobiophys.* In press.
24. Cooper, A. 1979. Energy uptake in the first step of visual excitation. *Nature (Lond.).* 282:531–533.