certain mammalian peptide hormones reflect the evolutionary conservation of compounds that are present in lower organisms and that may subserve some function therein. A variety of peptide hormones has been identified in certain protozoa or fungi, and insulin and secretin have been identified in the prokaryote *Escherichia coli* (*16*). Seemingly analogous to our findings are those of Richert and Ryan (*17*), who identified a saturable binding site for the glycopeptide hormone hCG in *Pseudomonas maltophilia* and our own finding of a binding site for bTSH in certain strains of *E. coli*. The functional significance, if any, of the bTSH binding site in *Y. enterocolitica* is uncertain, as is that of the hCG binding site in *Pseudomonas*, but remains to be evaluated.

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Fourier Transform Infrared Difference Spectra of Intermediates in Rhodopsin Bleaching

Abstract. *The membrane protein rhodopsin is the primary light receptor in vision. Fourier transform infrared difference spectroscopy is sensitive to conformational changes in both the protein and the retinylidene chromophore of rhodopsin. By blocking rhodopsin bleaching at specific intermediates, it is possible to elucidate some of the primary molecular events of vision.*

The central problem in vision research is understanding how light absorption by a single rhodopsin molecule in the photoreceptor membrane leads to neural excitation (*1, 2*). Despite extensive research on the primary photochemistry and molecular mechanisms in rhodopsin, only limited information is available. This problem is typical of the difficulty of obtaining information about the structure and conformational changes of membrane proteins involved in important cellular processes such as ionic transport.

We report here on the application of Fourier transform infrared (FTIR) difference spectroscopy to study the conformational changes that occur in the photoreceptor membrane. As demonstrated with purple membrane from *Halobacterium halobium* (*3–5*), alterations of individual groups in the bacteriorhodopsin molecule can be detected with this new method. Previous FTIR studies of the photoreceptor membrane have focused on the secondary structure of rhodopsin and the orientation of the rhodopsin α-helices (*6*). Kinetic infrared spectroscopy has revealed time-dependent changes in the infrared bands of photoreceptor membrane on light exposure (*7*). This approach can be used to obtain the infrared difference spectrum of the metarhodopsin (meta) I and II transitions, although a new sample is required for each data point (*7*). We have found that changes in both the protein and the chromophore of rhodopsin at different stages of bleaching can be easily detected by FTIR difference spectroscopy. Specific findings include confirmation that an isomerization of the chromophore oc-

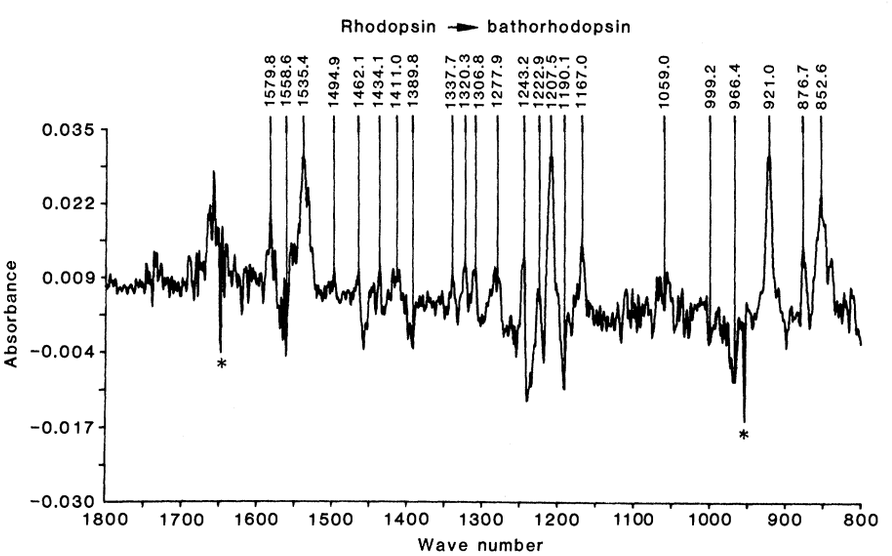

Fig. 1. Fourier transform difference spectra of photoreceptor membrane film deposited on AgCl, measured at 77 K while fully humidified. Photoreceptor membrane was isolated from bovine retinas (*24*), and films were formed by the isopotential spin-dry method (*25, 26*). Films had an absorbance of 0.4 to 0.5 at 500 nm and appeared optically transparent. Films were humidified by exposing a blank AgCl window to saturated water vapor while cooling the back of the window with a flow of cold N_2 gas for 15 to 30 seconds, then immediately placing the window in a cell containing an identical window with the deposited photoreceptor membrane film and sealing the cell. The amount of water vapor present was monitored from the H_2O bands at 3400 cm^{-1}. The cell was mounted on the copper tail of a low-temperature dewar (Janis Research Company, Stoneham, Massachusetts) equipped with a KBR and Ge window. The FTIR measurements were made with a Nicolet MX-1 spectrometer at a resolution of 2 cm^{-1}. Difference spectra were obtained by subtracting a reference spectrum recorded in the dark from a sample spectrum after 5 minutes of illumination with 500-nm light from a 600-W incandescent source filtered with Kodak Wratten filter 47. The reference and sample spectra were recorded with 300 scans (a total of 5 minutes). Four spectra from different samples were co-added to yield the spectrum shown. The asterisks mark peaks due to instrument-related artifacts.

curs by the first photoproduct, bathorhodopsin. By meta II, conformational change has spread to the water-accessible region or regions of the protein and involves glutamate or aspartate or both. However, a major rearrangement in the secondary structure or orientation of rhodopsin in the membrane can be excluded.

Figure 1 shows the rhodopsin-to-bathorhodopsin difference spectrum calculated from FTIR spectra before and after illumination of a sample cooled to 77 K, which blocks the decay of bathorhodopsin (*8*). The positive peaks represent the formation of bathorhodopsin and the negative peaks the loss of rhodopsin. From these data, we conclude that the measurements are sufficiently sensitive to detect single group modes of the retinal chromophore. In particular, the three positive peaks at 853, 877, and 921 cm^{-1} can be assigned on the basis of resonance Raman spectroscopy, which selectively enhances the vibrations of only the rhodopsin chromophore (*9–15*), to the bathorhodopsin hydrogen out-of-plane bending modes of the C-14, C-10, and C-11 retinal chain hydrogens, respectively (*11*). The negative peak at 966 cm^{-1} also agrees with the resonance Raman assignment of a hydrogen out-of-plane bending mode in dark-adapted rhodopsin (*11*). Hence, the vibrational changes occurring in the rhodopsin chromophore can be easily measured with this method, which does not rely on the use of a perturbing visible probe beam as does the Raman method. Furthermore, with polarized FTIR it should be possible to determine the orientation of specific chromophore groups relative to the membrane plane (*6*).

Peaks in the "fingerprint" region from 1100 to 1400 cm^{-1} also appear, on the basis of resonance Raman spectroscopy (*10–13*), to be of chromophoric origin. For example, the positive peaks at 1167, 1208, 1223, 1243, and 1278 cm^{-1} closely match the peaks observed in bathorhodopsin by the Raman method (*14, 15*). Similarly, the negative peaks at 999, 1190, 1216, 1240, and 1450 cm^{-1} correspond to Raman vibrations in rhodopsin (*14, 15*). There is also general agreement between the relative position of these peaks and the respective resonance Raman spectra in this region of the 11-*cis* and all-*trans* *n*-butylamine protonated derivatives of retinal (*16*). It is interesting that the reported infrared spectra of these model compounds (*7*) do not agree as well as resonance Raman spectra, possibly due to differences in the solvent used. We also note that the peaks at 1535 and 1559 cm^{-1} can be assigned to the ethylenic stretching (C=C) vibrations of bathorhodopsin and rhodopsin, respectively. However, the broad peaks above 1600 cm^{-1} were found to be artifacts due to drifts in the baseline. Hence, we conclude that most of the vibrational changes in the rhodopsin-to-bathorhodopsin transition can be assigned to the chromophore and are consistent with an isomerization from 11-*cis* to all-*trans*, as previously determined on the basis of resonance Raman spectra (*11, 14*).

In contrast, rhodopsin-to-meta II difference spectrum shown in Fig. 2A provides direct evidence for protein conformational changes. In particular, the peaks at 1767 and 1749 cm^{-1} can be assigned to the COOH groups of asparatate or glutamate residues on the basis of the frequency (*17, 18*) and the downshift of these peaks to 1757 and 1740 cm^{-1} when the bleaching is done in D_2O-humidified films (Fig. 2B) (*19*). A similar downshift D_2O humidification was observed for the peak at 1760 cm^{-1} in the FTIR difference spectrum of the bR570-to-M412 transition in bacteriorhodopsin (*3, 5*). Although this frequency is high relative to the 1740 cm^{-1} C=O stretch frequency measured in polybenzylasparatate, a shift to this high a frequency can occur in COOH groups with a lowered pK_a (*18*). A similar origin for the 1767 and 1749 cm^{-1} peaks is likely in the photoreceptor membrane. A change in effective protonation or hydrogen bonding for one or more COOH groups could give rise to the differences observed. Since the vibrational frequency of these groups is influenced by D_2O, we surmise that they are located in a water-accessible portion of rhodopsin. Furthermore, some hydrogen-deuterium exchange must occur before bleaching since the peak at 1767 cm^{-1} is assigned to the dark-adapted state of rhodopsin. The fingerprint region of the rhodopsin-to-meta II difference spectrum is also quite different from this region in the rhodopsin-to-bathorhodopsin difference spectrum, possibly due to nonchromophoric changes or to a change in the chromophore conformation compared to that in bathorhodopsin. Studies with isotopically labeled retinal will be necessary to decide between these possibilities. Also, the rhodopsin-to-meta II FTIR difference spectrum is consistent with the results of infrared kinetic spectroscopy (*7*), even though the latter results are at lower resolution.

The extent of conformational change in rhodopsin can be estimated from the fact that the total integrated intensity of the differences in the amide I region (1630 to 1680 cm^{-1}) is less than 1 percent of the total integrated intensity of the amide I peak. This peak in photoreceptor membrane films is linearly dichroic (*20, 21*), indicating that rhodopsin contains α-helices oriented predominantly perpendicular to the membrane plane. This con-

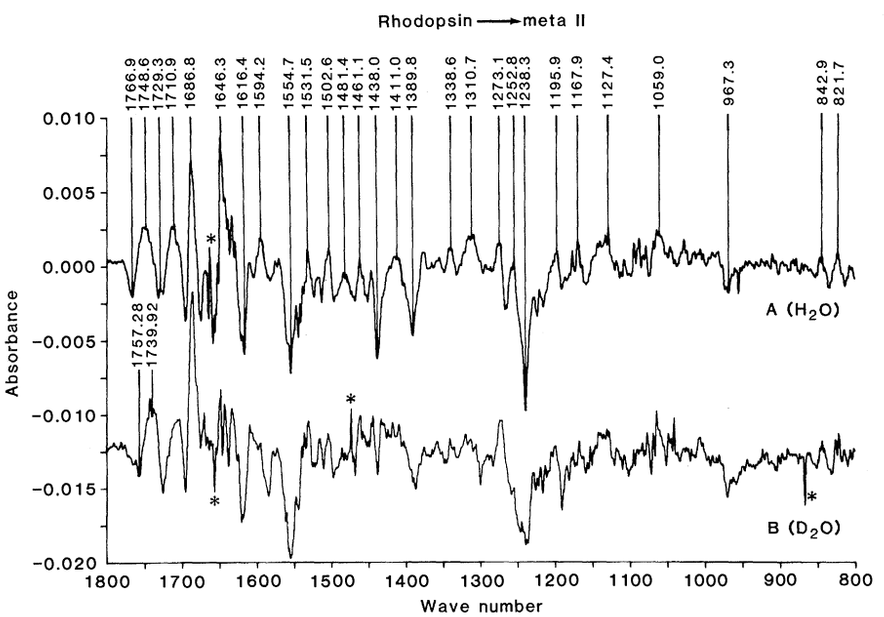

Fig. 2. Fourier transform infrared difference spectra of photoreceptor membrane film prepared as described in Fig. 1 at room temperature. (A) Rhodopsin to meta II difference spectrum of H_2O-humidified films. Reference and sample spectra were recorded with 300 scans (5 minutes). A light of wavelength 530 nm (Kodak Wratten filter 12) illuminated the sample for 5 minutes between reference and sample scans. Visible absorption was measured before and after bleaching on a Cary 219 visible-ultraviolet absorption spectrometer. (B) Same as (A) but with D_2O-humidified films.

clusion has been supported by the complete amino acid sequence of rhodopsin (*22*). If the nonhelical content of rhodopsin is taken into account, these helices should be oriented on average less than 30° from the membrane normal (*21*). We estimate that an average change of more than 5° would result in more than a 1 percent absorption change in the amide I region and conclude that 5° is the limit of orientational change of rhodopsin α-helices relative to the membrane on bleaching to meta II. In addition, a conformational change that resulted in a significant alteration of the secondary structure of rhodopsin would result in a much larger difference than we observed. Such a conformational change is also inconsistent with the results of ultraviolet absorption and circular dichroism measurements (*23*).

In conclusion, FTIR difference spectroscopy can provide detailed information about molecular changes in photoreceptor membrane. The effects of blocking rhodopsin bleaching at other intermediates such as meta I and the effects of deuterium-hydrogen exchange in the protein and chromophore will be reported subsequently. Further studies with chemically modified and reconstituted rhodopsin should be useful for identifying specific groups and their role in visual transduction. The techniques used here should also be applicable to other membrane-based systems, for instance, to study the active transport of calcium.

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Increased Sensitivity of Lymphocytes from People over 65 to Cell Cycle Arrest and Chromosomal Damage

Abstract. *Flow cytometry revealed that, in the presence of tritiated thymidine, a greater percentage of phytohemagglutinin-stimulated lymphocytes from old human donors were arrested in the G_2 or M phase than were cells from young donors. Furthermore, lymphocytes from old donors showed significantly more chromosomal damage than did lymphocytes from young donors. Lymphocyte cultures from old or young donors not exposed to tritiated thymidine had the same percentage of cycling lymphocytes in G_2 or M, although the number of lymphocytes stimulated by phytohemagglutinin to enter the cell cycle was significantly lower in cultures from old donors. Thus, the impaired incorporation of tritiated thymidine by phytohemagglutinin-exposed lymphocytes from old humans reflects both an impaired proliferative response to phytohemagglutinin and an increased sensitivity to the radiobiological effects of tritiated thymidine.*

When lymphocytes from aged humans and experimental animals are incubated with phytohemagglutinin (PHA), they incorporate less [^{3}H]thymidine than do lymphocytes from young donors (*1–3*). This has been attributed to an impaired proliferative capacity of lymphocytes from old donors, resulting from a paucity of mitogen-responsive T lymphocytes and from a failure of mitogen-responsive T lymphocytes to divide repeatedly in culture (*4, 5*).

Pollack *et al.* (*6*) reported that the use of [^{3}H]thymidine incorporation to assess lymphocyte proliferation in culture may be flawed. Using flow cytometry, these investigators found that the percentage of PHA-stimulated human lymphocytes in phase G_2 or M was significantly increased by exposure to [^{3}H]thymidine. This arrest of the cell cycle was secondary to the radiobiological effect of [^{3}H]thymidine. Neither tritiated water (which is not concentrated in the nucleus) nor thymidine-induced cell cycle arrest. It was suggested that the nucleus is the site of damage caused by [^{3}H]thymidine. The inability of cells to repair DNA damage induced by [^{3}H]thymidine may have resulted in the accumulation of lymphocytes in the G_2 or M phase.

Since cells from old donors are impaired in their capacity to repair DNA (*7*), it is possible that the reduced incorporation of [^{3}H]thymidine by lymphocytes from old as compared to young subjects is due at least in part to an increased sensitivity of lymphocytes from old donors to the radiobiological effects of [^{3}H]thymidine. We therefore determined the sensitivity of lympho-

Table 1. Cytogenetic analysis of PHA-stimulated lymphocytes exposed to [^{3}H]thymidine. Chromosomal preparations were prepared by adding Colcemid (0.05 μg/ml) to the cultures 1 hour before harvest. Ten milliliters of 0.075*M* KCl was added to each of the cell pellets, which were then incubated for 8 minutes at 37°C, fixed in methanol and glacial acetic acid (3:1), and washed three times in fixative. The final cell suspensions were dropped onto wet slides. Slides were stained with 3 percent Giemsa and coded for blind analysis. Metaphase figures derived from PHA-stimulated whole blood cultures exposed to [^{3}H]thymidine (0.1 μCi/ml) were analyzed. Cells were scored as abnormal if they contained exchange figures, chromosome breaks, or chromosome fragments.

Subject	Number of metaphase figures	
	Abnormal	Normal
Old		
1	17	8
2	14	11
3	10	3
Mean	13.67	7.33
Young		
1	9	16
2	9	16
3	10	15
Mean	9.33	15.66