Tryptophan in Bovine Rhodopsin: Its Content, Spectral Properties, and Environment†

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ABSTRACT: The tryptophan content of purified bovine rhodopsin was obtained by two independent methods: direct analysis of hydrolysates prepared by digestion of opsin with methanesulfonic acid containing 0.2% 3-(2-aminoethyl)indole and a computer-assisted analysis of the near-UV spectrum of rhodopsin. Both methods gave a value of eight tryptophan residues per rhodopsin. Based on the near-UV spectral analysis, the light-induced difference spectrum of rhodopsin, and the susceptibility of residues to oxidation by *N*-bromosuccinimide, we concluded that approximately half of the tyrosine and tryptophan residues are shielded to some extent from the aqueous solvent, that two of the tryptophan residues are in very apolar environments, and that following light excitation at least one of these tryptophan residues and several tyrosines are exposed to an aqueous environment. Analysis of rhodopsin absorption in the far-UV indicated that below 240 nm, approximately half of the absorption is due to aromatic residues and that the other half is largely due to the peptide bond. The effect of illumination on secondary structure is to induce a loss in helical structure, calculated to involve 35% of the amino acid residues in purified rhodopsin. If light-induced changes in secondary structure are specifically excluded, most of these results can be extended to bovine rod outer segment membranes.

Considerable uncertainty exists concerning the content of aromatic amino acids, especially of tryptophan, in the visual pigment rhodopsin. A variety of analytical methods have been employed: spectral analysis of UV[1] absorption (Collins & Morton, 1950; Hubbard, 1969), titration with *N*-bromosuccinimide (Heller, 1968; Robinson et al., 1972; Cooper & Hogan, 1976), and amino acid analysis of toluenesulfonic acid hydrolysates of opsin (Trayhurn et al., 1974). The reported number of tryptophan residues per mole of rhodopsin varies widely from 3 (Trayhurn et al., 1974) to 15 (Hubbard, 1969).

In this study we determined the tryptophan content of highly purified bovine rhodopsin by two independent procedures. The first consisted of direct amino acid analysis of opsin hydrolysates obtained by incubation of opsin with methanesulfonic acid containing 0.2% 3-(2-aminoethyl)indole. This method of protein hydrolysis was reported to have little destructive effect on tryptophan (Simpson et al., 1976). The second procedure consisted of deconvolution of the UV absorption spectrum of rhodopsin into its component parts. This was accomplished by a computer-assisted curve-fitting method in which reference spectra of phenylalanine, tyrosine, and tryptophan were compared to the experimental spectrum of rhodopsin between 257 and 320 nm.

We also investigated the local environments of the aromatic residues of rhodopsin with particular emphasis on tryptophan since several workers have suggested that a specific tryptophan is near the binding site of the retinal prosthetic group and may interact with the polyene chain (Kropf et al., 1973; Copper & Hogan, 1976; Rafferty, 1979; Chabre & Breton, 1979). The approach we used involved both a chemical and a spectroscopic procedure. In the chemical procedure, we investigated the accessibility of various residues to the aqueous solvent by incubation of purified rhodopsin with the oxidizing agent *N*-bromosuccinimide. In the spectroscopic procedure, we included possible bathochromic shifts of the near-UV absorption bands of the model aromatic amino acids in the curve-fitting method mentioned above. Conclusions were then drawn concerning the polarity and polarizability of the local environments of the aromatic residues of rhodopsin based on the extent of the inferred bathochromic spectral shifts of these residues (Wetlaufer, 1962; Donovan, 1969). We also measured the light-induced difference absorption spectrum of purified rhodopsin in the UV. From a similar spectroscopic analysis, conclusions were drawn about light-induced changes in the environments of tyrosine and tryptophan residues.

Finally, we used the experimental amino acid composition to analyze the contributions of the different amino acid side chains to the absorption spectrum of rhodopsin in the region between 185 and 240 nm. We found that approximately half of the absorption is due to aromatic residues and that the other half is largely due to peptide bonds.

Materials and Methods

Purification of Bovine Rhodopsin. Rod outer segments were prepared from frozen bovine retinas (purchased from George A. Hormel Co., Austin, MN) as described previously (Shichi et al., 1969). Rhodopsin was extracted with 1% Emulphogene BC720 (a generous gift from GAF Corp., NY) in 67 mM potassium phosphate, pH 6.5, and purified on a calcium phosphate–Celite column as described (Shichi et al., 1969). Rhodopsin-containing fractions (A_{278}/A_{498} = 1.6–1.8) were pooled and frozen at −30 °C until ready for use. The purified rhodopsin was homogeneous as evaluated by the following criteria. (1) The purified rhodopsin was eluted as a homogeneous protein peak from a calcium phosphate–Celite column and a ECTEOLA-cellulose column as was reported (Shichi et al., 1969). (2) The purified rhodopsin migrated as a single band in sodium dodecyl sulfate gel electrophoresis (data not shown). (3) The spectral ratio (A_{278}/A_{498}) for homogeneous rhodopsin samples prepared by different methods has been reported to be 1.7 ± 0.1 (Shichi, 1970; Hong & Hubbell, 1972; Albert & Litman, 1978). Rhodopsin preparations used in the present work showed the spectral ratio in the range of 1.6–1.8. (4) If purified rhodopsin was tested by Ouchterlony against antirhodopsin I_gG, it gave a single precipitin line (data not shown). Rhodopsin purified by our method was recently shown to be homogeneous in immunoelectrophoresis (Blaustein & Dewey, 1979).

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[1] Abbreviations used: UV, ultraviolet; *A*, absorbance; Phe, phenylalanine; Tyr, tyrosine; Trp, tryptophan; NRO, *N*-retinylideneopsin.

Retinal and Protein Determinations. *all-trans*-Retinal was reacted with hydroxylamine as described (Shichi, 1970), and the concentration of retinal oxime formed was determined on a basis of $\epsilon_{362nm} = 59.3 \times 10^3$ L cm^{-1} mol^{-1}. Protein was determined with Folin phenol reagent (Lowry et al., 1951).

Amino Acid Analysis. Opsin protein which had been dialyzed until free of detergent and lyophilized (0.2–0.3 mg) was hydrolyzed in vacuo at 115 °C for 24, 48, 72, and 96 h with 1.0 mL of 4 N methanesulfonic acid containing 0.2% 3-(2-aminoethyl)indole (Simpson et al., 1976). After hydrolysis, the hydrolysates were partially neutralized with 3.5 N NaOH, filtered, and provided for amino acid analysis on a Beckman automatic amino acid analyzer, Model 121. For determination of the half-cystine content of opsin, dialyzed opsin protein was oxidized with performic acid by the method of Hirs (1956) and cysteic acid was determined with an amino acid analyzer.

Polyacrylamide Gel Electrophoresis. Purified rhodopsin was dialyzed at 20 °C for 24 h against 10 mM Tris–acetate buffer (pH 7.4) containing 2.5% sodium dodecyl sulfate and 40 mM dithiothreitol, and electrophoresis was carried out on an 8% polyacrylamide gel containing 2.5% sodium dodecyl sulfate as described by Fairbanks et al. (1971). The gel was stained with Coomassie Blue for detection of protein. Molecular weight markers (BDH Biochemicals) were used for determination of the molecular weight of rhodopsin.

Modification of Rhodopsin with N-Bromosuccinimide. Purified rhodopsin was incubated with 75-fold *N*-bromosuccinimide at pH 4 for a given time (Ramachandran & Witkop, 1967). The reaction mixture was brought to pH 10 with NaOH and immediately dialyzed against a large volume of distilled water at 3 °C. Oxidation of amino acid residues could have proceeded to some extent during dialysis.

Spectral Measurements and Analysis. Absorption spectra of rhodopsin, phenylalanine (Phe), tyrosine (Tyr), and tryptophan (Trp) were recorded with either a Cary 14 or a Cary 17 spectrophotometer at 25 °C to wavelengths as short as 185 nm. For recordings below 200 nm, the spectrophotometer was purged with N_2. The solvent for purified rhodopsin was 1% Emulphogene BC720 in 67 mM potassium phosphate buffer (pH 6.9). The spectra of the aromatic amino acids (Sigma Chemicals) were obtained by using two solvents, the same detergent–buffer system and buffer only (10 mM phosphate buffer, pH 6.9). Spectral differences due to solvent were negligible; bathochromic shifts of no more than 2 Å were observed in detergent–buffer. The light-induced difference absorption spectrum of purified rhodopsin was obtained as previously described (Rafferty, 1979). Rhodopsin was illuminated with orange light (Kodak Gelatin Filter No. 22). Data manipulations, the curve-fitting procedure, and graphical designs were carried out using the MLAB program developed by the Division of Computer Research and Technology at the National Institutes of Health. Curve fitting in MLAB is based on minimizing a sum of squares value using the Marquardt–Levenberg method (Marquardt, 1963).

Results

Molecular Weight of Rhodopsin. Four different preparations of highly purified opsin were analyzed by polyacrylamide gel electrophoresis in 2.5% sodium dodecyl sulfate. A linear relationship was obtained between the logarithm of molecular weight and mobility of the standard marker proteins. Interpolation gave an average molecular weight for rhodopsin of 38 000 ± 1000. The protein in each preparation migrated as a single band, although polymeric forms of opsin were occasionally detected. Heat treatment of opsin in sodium dodecyl sulfate and prolonged dialysis seemed to accelerate the polymerization. Retinal and protein determination on the purified preparation gave rise to an average molecular weight of 39 000 ± 1700.

Table I: Amino Acid Composition of Opsin

amino acid	molar ratio[a] after hydrolysis for 24 h	48 h	72 h	96 h	probable no. of residues
lysine	10.0	10.0	10.0	10.0	10
histidine	4.6	4.9	4.6	4.7	5
arginine	5.6	6.4	5.7	6.3	6
aspartic acid	21.7	21.5	22.1	21.7	22
threonine	25.2	24.8	23.2	21.2	25
serine	13.0	12.1	11.2	11.0	13
glutamic acid	28.2	29.3	28.7	28.9	29
proline	19.6	20.5	20.8	20.1	20
glycine	24.7	21.7	23.3	23.8	24
alanine	27.4	27.2	27.7	27.8	28
half-cystine	11.8	11.3	11.5	—	12
valine	22.3	24.0	26.6	28.2	28
methionine	9.7	11.9	12.3	11.7	12
isoleucine	11.6	12.4	15.1	14.7	15
leucine	17.2	19.9	22.0	21.2	22
tyrosine	15.4	16.6	16.7	16.8	17
phenylalanine	25.6	26.1	27.8	28.1	28
tryptophan	7.9	8.4	7.8	7.9	8
				total:	324

[a] Lysine was taken as 10. Other numbers represent averages of four determinations.

Amino Acid Analysis of Opsin. When opsin was hydrolyzed with methanesulfonic acid containing 0.2% 3-(2-aminoethyl)indole, all tryptophan residues were liberated in 24 h and were not destroyed by hydrolysis for as long as 96 h (Table I). From the analysis and on the basis of a trial molecular weight of 39 000 for rhodopsin, 8 tryptophans, 17 tyrosines, and 28 phenylalanines were determined per mol of rhodopsin. The half-cystine content of performic acid treated opsin was found to be 12. The structure of the carbohydrate moiety of rhodopsin was recently elucidated (Liang et al., 1979; Fukuda et al., 1979). The N-terminal methionine of the pigment was found to be acetylated (Tsunasawa et al., 1980). From the amino acid composition and other structural information, a molecular weight of 38 720 is estimated for bovine rhodopsin.

Absorption and Difference Absorption Spectra. The absorption spectrum of bovine rhodopsin between 185 and 650 nm is shown in the lower half of Figure 1 (parts d–f); the light-induced difference absorption spectrum, recorded over the same spectral range, is shown in the upper half of Figure 1 (parts a–c). Spectral data are collected in Table II. Absorption is given in units of molar extinction and was calculated by setting the measured absorbance at 498 nm (Figure 1f) equal to 40.6×10^3 L mol^{-1} cm^{-1} (Wald & Brown, 1953). The near-UV spectrum of rhodopsin (Figure 1e) arises primarily from π–π^* transitions of the aromatic residues, particularly those of Tyr and Trp (Shichi et al., 1969; Rafferty et al., 1977; Rafferty, 1979). Phe residues make a secondary contribution as indicated by the slight, fine structure at 254, 260, 265, and 270 nm. The retinal prosthetic group also makes a significant though secondary contribution between 250 and 300 nm. This contribution was approximated by fitting a single Gaussian function to the β band centered at 350 nm and then extrapolating the fitted function into the near-UV. This approximation is shown by the dashed curve in Figure 1e. The far-UV spectrum (Figure 1d) is associated primarily with π–π^* transitions of both the aromatic residues and the peptide bond (amide transitions) (Shichi et al., 1969; Rafferty et al., 1977; Rafferty, 1979). Contributions from retinal, the carbohydrate moiety, and other amino acid side chains are probably quite

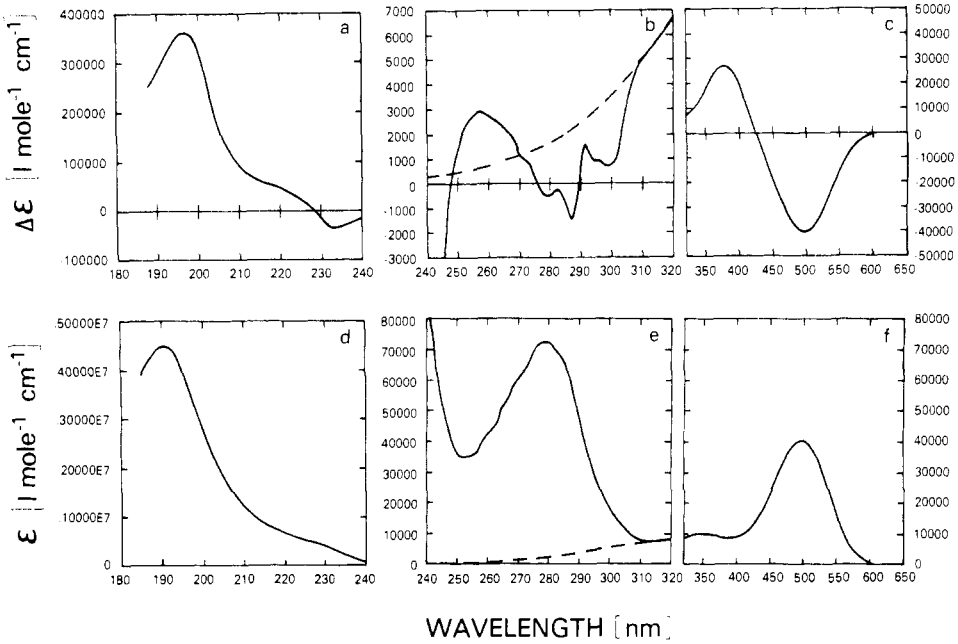

FIGURE 1: Absorption and light-induced difference absorption spectra of purified bovine rhodopsin in 1% Emulphogene BC720 at 25 °C and pH 6.9. The absorption spectrum shown in the lower half of the figure (solid lines in parts d–f) is that of unilluminated rhodopsin. The difference spectrum shown in the upper half of the figure (solid lines in parts a–c) is that of illuminated minus unilluminated samples recorded ~60 min after illumination with orange light. The dashed lines in parts b and e show Gaussian extrapolations of adjacent bands in the visible region.

Table II: Data of the Experimentally Based Absorption and Difference Absorption Spectra of Purified Bovine Rhodopsin: Wavelengths of Extrema and Crossovers [λ (nm)], Molar Extinction Coefficients [ϵ_{mol}[a] (L cm^{-1} mol^{-1})], and Changes in Molar Extinction Coefficients [$\Delta\epsilon_{mol}$[a] (L cm^{-1} mol^{-1})][b]

absorption spectrum				difference absorption spectrum			
original[c]		corrected[d]		original[c]		corrected[e]	
λ	ϵ_{mol}	λ	ϵ_{mol}	λ	$\Delta\epsilon_{mol}$	λ	$\Delta\epsilon_{mol}$
498	40 600			500	−40 000		
				424	0		
350	10 340			380	27 400		
						299.7	−2700
						294[f]	−1900
				291.3	1 630		
				289.1	0		
286[f]	65 000	286[f]	61 700	287.2	−1 430	287.4	−3700
279.5	72 500	279.5	70 100	278.7	−470	279.3	−2150
270[f]	60 600	270[f]	59 200	276.2	0	270.2	0
265[f]	51 600	265[f]	50 600	267[f]	2 100	267[f]	1000
260[f]	43 000	260[f]	42 300	257.2	3 000	257.1	2200
254[f]	35 900	254[f]	35 500	252[f]	2 300	252[f]	1900
				247.8	0		
				233.2	−34 900		
228[f]	470 000			227.8	0		
				221[f]	42 000		
191.0	4 510 000			196.5	360 000		

[a] All extinction data were normalized so that ϵ_{mol} = 40 600 L cm^{-1} mol^{-1} at 498 nm. [b] Spectral data in the region 250–300 nm are tabulated both for the original spectra (Figure 1, solid lines) and for corrected spectra obtained by subtraction of the extrapolated Gaussian curves shown in Figure 1, parts b and e. These corrected spectra are designated "experimental" in Figures 2 and 3. [c] Figure 1, solid lines. [d] Figure 2, "experimental". [e] Figure 3, "experimental". [f] Shoulder.

small. The light-induced difference spectrum (illuminated minus unilluminated samples) was recorded ~60 min after illumination; changes in the far-UV difference spectrum persisted for 45 min after illumination; after 45 min the absorbance at 197 nm was maximal and stable within 5% for the period required to record the complete difference spectrum. The visible difference spectrum (Figure 1c) arises from the bleaching of rhodopsin (negative band centered at 500 nm) and the appearance of *N*-retinylideneopsin (NRO). NRO was identified as the product of illumination by alternatively adding base and acid to the sample and observing the reversible appearance of the deprotonated form (NRO_{380}, λ_{max} = 380 nm) and the protonated form (NRO_{440}, λ_{max} = 440 nm). The size of the positive band centered at 380 nm in Figure 1c indicates that NRO_{380} predominates under the conditions of the measurement (60 min after illumination at 25 °C in 1% Emulphogene, pH 6.9). The near-UV difference spectrum (Figure 1b) has the appearance of a positively displaced aromatic residue perturbation spectrum (Donovan, 1969). The obvious source of the positive displacement is an overlap of the 380-nm band into the near-UV region. The extent of this displacement was approximated by fitting a single Gaussian function to the short wavelength segment of the visible NRO_{380} spectrum and extrapolating this function into the near-UV. The extrapolated function is shown in Figure 1b as a dashed curve. The far-UV difference spectrum (Figure 1a) is similar to that of a protein helix–coil transformation (Gratzer, 1967). Expected contributions from aromatic residue perturbations are probably of secondary importance below 210 nm.

Analysis of the Near-UV Spectrum. The experimental absorption spectrum of bovine rhodopsin (Figure 1e) was corrected for β-band absorption in the near-UV by subtraction of the extrapolated Gaussian curve. Reference absorption spectra of Phe, Tyr, and Trp were then fitted to the corrected spectrum of rhodopsin according to the following model. At any given wavelength

$$\epsilon^{calcd} = A\epsilon_2^{Phe} + \sum_{i=0}^{10}(B_i\epsilon_i^{Tyr} + C_i\epsilon_i^{Trp})$$

ϵ^{calcd} is the calculated molar extinction which is compared to the experimental extinction of rhodopsin throughout a particular spectral region. ϵ_2^{Phe}, ϵ_i^{Tyr}, and ϵ_i^{Trp} are the molar extinctions of the aromatic residue reference spectra. For each aromatic amino acid, reference spectra consisted of the absorption spectrum measured in an aqueous buffer (pH 6.9) and additional spectra generated by translation of the aqueous

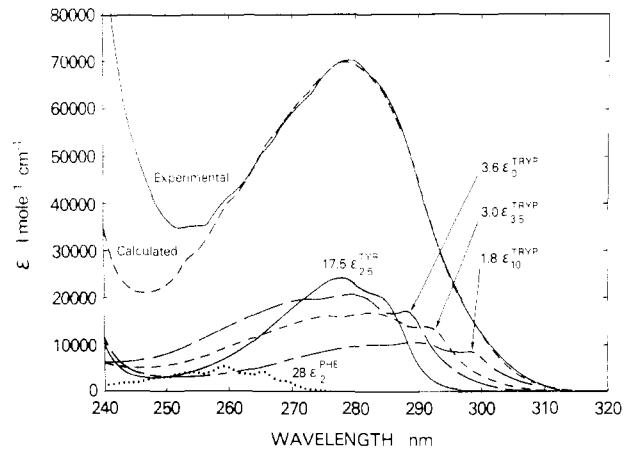

FIGURE 2: Comparison of the experimental absorption spectrum of purified bovine rhodopsin in the near-UV to a spectrum calculated by a least-squares procedure as the sum of the spectra of the free aromatic amino acids. The components of the calculated spectrum are also shown. Several of these spectra represent bathochromically shifted species of the aromatic amino acids. See the text for details. The experimental spectrum was taken from Figure 1e and corrected for overlap of bands located in the visible region by subtraction of the extrapolated Gaussian curve.

spectrum along the wavelength scale, in the direction of longer wavelengths, at intervals of 1 nm. Thus, the index, i, gives the number of nanometers that a particular reference spectrum is shifted to the red compared to the aqueous spectrum. The coefficients A, B_i, and C_i simply give the number of residues in each spectral category. The rationale for this multiplicity of reference spectra is to better model the effects of apolar interior protein environments which are known to produce bathochromic shifts in aromatic residue spectra (Wetlaufer, 1962; Gratzer, 1967; Donovan, 1969). In the computer-assisted fitting procedure, the coefficients B_i and C_i were varied so as to minimize the sum of squares of the difference between the calculated and experimental extinctions between 257 and 320 nm. Because of the low molar extinction of Phe residues in this spectral interval ($\epsilon^{Phe} < 200$), estimation of A by the curve-fitting method was highly imprecise. A was therefore assumed to be 28, the number of Phe residues determined in the analysis of hydrolysates. However, comparison of the fine structure in the rhodopsin spectrum between 250 and 270 nm to the aqueous spectrum of Phe indicated that many of the Phe residues occupy apolar sites in rhodopsin. The best correspondence was obtained by assuming that the spectra of all 28 residues are bathochromically shifted 2 nm. (An equivalent distribution is that 14 residues are shifted 0 nm and that 14 residues are shifted 4 nm.) On the basis of this analysis, a single reference spectrum for Phe was used in the curve-fitting model, a spectrum shifted 2 nm to the red, ϵ_2^{Phe}.

The results of the curve-fitting procedure are shown graphically in Figure 2. A minimum sum of squares condition was obtained which gave the number of Tyr and Trp residues associated with each spectral category. The distribution of Tyr residues was 4.78 Tyr_2 (Tyr spectrum shifted 2 nm) and 12.76 Tyr_3 (spectrum shifted 3 nm). The distribution of Trp residues was 3.63 Trp_0 (aqueous Trp spectrum), 1.07 Trp_3 (spectrum shifted 3 nm), 1.89 Trp_4 (spectrum shifted 4 nm), and 1.84 Trp_{10} (spectrum shifted 10 nm). All other values of B_i (except $i = 2$ and 3) and C_i (except $i = 0$, 3, 4, and 10) were zero or nearly zero. The total number of Tyr residues required for the minimum sum of squares condition was 17.54; the total number of Trp residues was 8.43; both values are in excellent agreement with the hydrolysate analysis. The calculated sum of the extinctions of the component aromatic acids is compared to the experimental rhodopsin spectrum in Figure 2. The correspondence of calculated and experimental spectra is excellent between 257 and 320 nm. Below 257 nm, amide transitions begin to contribute appreciably to the experimental spectrum. The components of the calculated spectrum are also shown in Figure 2.

We explored the uniqueness of this particular distribution of aromatic residue reference spectra by setting constraints on selected coefficients, B_i and C_i, during the curve-fitting procedure. We concluded the following. (1) The range of possible distributions was greater for Tyr than for Trp while still maintaining a good though slightly less precise correspondence between the calculated and experimental spectra. For example, an acceptable alternative distribution for Tyr was 5.50 Tyr_0 (aqueous spectrum) and 12.21 Tyr_4 (spectrum shifted 4 nm). It is also possible that a small number of Tyr residues (≤ 2) might exist as highly red shifted species (spectra shifted more than 5 nm). However, the data cannot accommodate much variation in the distribution of Trp spectra. Three categories of spectra are required: nonshifted, shifted 3–4 nm, and highly red shifted 8–10 nm. An invariant requirement for a good fit is that the spectra of two Trp residues are highly red shifted. (2) More than half of the Tyr residues and more than half of the Trp residues have spectra which are shifted 3 nm or more. (3) The total number of Tyr and Trp residues required for a good fit is subject to little error: 17.5 $\pm$ 0.5 for Tyr and 8.4 $\pm$ 0.2 for Trp.

Analysis of the Near-UV Difference Spectrum. As a working hypothesis, we assumed that the light-induced near-UV difference spectrum of rhodopsin (Figure 1b) arises from a transfer of one or more aromatic residues from an interior apolar environment to an aqueous environment. We also assumed that the spectra of aromatic residues in an apolar environment are adequately represented by red-shifted aqueous spectra and consequently that transfer to an aqueous environment results in a corresponding blue shift. Therefore, we compared model difference spectra, defined as the difference in extinction between nonshifted and red-shifted species ($\epsilon_0 - \epsilon_i$) of Tyr and Trp, to the experimental rhodopsin difference spectrum. Potential contributions by Phe to the rhodopsin difference spectrum were discounted based on the low extinction and inappropriate extinction maxima of Phe above 250 nm. The experimental near-UV difference spectrum was first corrected for overlap of the positive 380-nm band by subtraction of the extrapolated Gaussian curve shown in Figure 1b. Because of the magnitude of this correction, the extinction values for the bands in the experimental difference spectrum should be taken only as approximate.

The results of this analysis are shown in Figure 3 The magnitudes and positions of the two long-wavelength negative bands near 300 and 294 nm are simulated fairly well by a model difference spectrum generated by a 10-nm blue shift of the spectrum of one Trp residue ($\epsilon_0^{Trp} - \epsilon_{10}^{Trp}$). An observation which confirms this assignment is that the 294-nm band is incompletely resolved from the 300-nm band in both the experimental and modeled spectra; difference spectra generated by shifting the Trp spectra less than 6 nm always show two well-resolved long-wavelength negative bands. The two shorter wavelength negative bands near 287 and 279 nm in the experimental spectrum are simulated fairly well by a difference spectrum generated by a 3-nm blue shift of the spectra of four Tyr residues, $4(\epsilon_0^{Tyr} - \epsilon_3^{Tyr})$.

Analysis of the Far-UV Spectrum. The contribution of the side chains of the amino acid residues of rhodopsin to the experimental far-UV spectrum was computed based on the

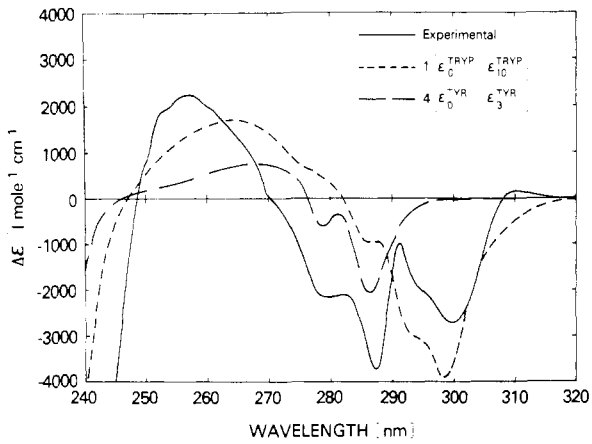

FIGURE 3: Comparison of the experimental light-induced difference spectrum of purified bovine rhodopsin in the near-UV to model difference spectra of tryptophan and tyrosine. The experimental difference spectrum was taken from Figure 1b and corrected for overlap of bands located in the visible region by subtraction of the extrapolated Gaussian curve. The model difference spectra were obtained for both tryptophan and tyrosine by subtracting the spectrum of the free amino acid in water from the spectrum obtained by translating the aqueous spectrum several nanometers to the red. See the text for details. The model spectra shown simulate the main features of the experimental difference spectrum of rhodopsin.

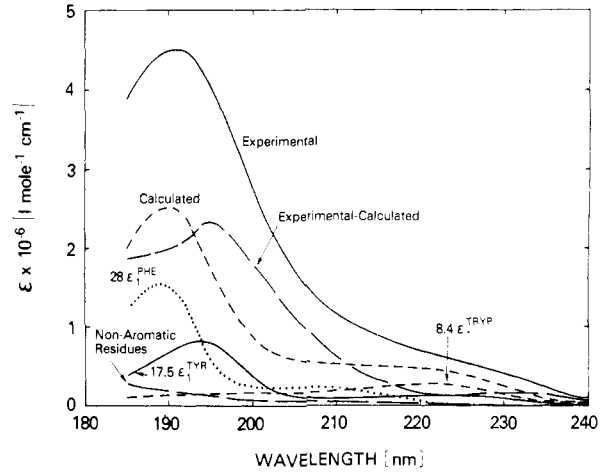

FIGURE 4: Deconvolution of the experimental far-UV absorption spectrum of purified bovine rhodopsin. The experimental spectrum was taken from Figure 1d. Composite absorption spectra associated with side chain absorption are shown for each aromatic amino acid and were calculated from the absorption of the free amino acids, corrected for carboxyl and amino absorption, and the number of aromatic residues in rhodopsin as determined in this study. The composite spectrum of the side chains of all the remaining amino acids is also shown. The calculated spectrum is the sum of the spectra of all of the amino acid side chains in rhodopsin. The difference of the experimental and calculated spectra should represent the contributions of the remaining far-UV chromophores, primarily the peptide bond. See the text for details.

amino acid content of rhodopsin as determined in this study. The spectra computed for the aromatic amino acids are shown in Figure 4 along with a spectrum which is the sum of the extinctions of the remaining nonaromatic residues. Absorption spectra for the aromatic amino acids in buffer were corrected for α-carboxyl and amino absorption and were translated 1 nm to the red as a crude approximation of the net effect of an apolar environment as suggested by the results of the near-UV spectral analysis. (A 2-nm bathochromic shift at 260 nm is approximately equivalent to a shift of 1 nm at 190 nm if spectra are translated on a wavenumber scale.) Reference spectra for the nonaromatic amino acids were those measured by McDiarmid (1965) and reported by Gratzer (1967). The spectrum designated as "calculated" in Figure 4 is the sum of the extinctions of all of the side chains in rhodopsin. Thus, approximately half of the far-UV absorption of rhodopsin is due to the amino acid side chains, particularly those of the aromatic residues. Below 200 nm, Phe and Tyr residues separately make larger contributions than the side chains of all the remaining amino acids combined. The shoulder present in the experimental spectrum between 210 and 240 nm is largely due to the absorption of Tyr and Trp residues. The "experimental – calculated" spectrum also shown in Figure 4 should represent the contribution of all the remaining far-UV chromophores. Since the contribution of the carbohydrate moiety of rhodopsin is expected to be negligible, we attribute most of this difference spectrum to π–π^* amide transitions. However, the exact shape and magnitude of the experimental – calculated spectrum is quite dependent on the exact value of the bathochromic shift used to model the influence of apolar protein environments on the spectra of Phe and Tyr residues. Translation of the model aromatic spectra 1 nm in either direction results in a change of magnitude in the difference spectrum of $\sim\pm$300 000 at 195 nm, an uncertainty of about 25%.

Analysis of the Far-UV Difference Spectrum. As noted previously, the spectral changes shown in Figure 1a are basically similar to those reported for the α helix $\rightarrow$ random coil transition in model polypeptides (Gratzer, 1967). However, significant contributions from perturbation of Tyr and Trp residues should be expected above 210 nm. On the basis of the magnitude and probable assignment of bands in the near-UV difference spectrum of rhodopsin (Figures 1b and 3) and the published solvent perturbation spectra of free aromatic amino acids between 200 and 300 nm (Donovan, 1969), changes below 250 nm in the rhodopsin spectrum due to aromatic residues were estimated. We conclude that the negative band near 233 nm and the positive shoulder near 220 nm are associated mainly with light-induced perturbation of Tyr and Trp residues. However, below 215 nm, positive changes associated with the amide transitions should predominate and below 205 nm should represent more than 90% of the observed molar extinction changes.

Since extinction changes below 205 nm are mostly due to the amide transitions and since the data are expressed in molar units, an estimate can be made of the fractional change in helical content induced by illumination, $\Delta f_H = f_H^{RHO} - f_H^{NRO}$, where f_H^{RHO} and f_H^{NRO} are the residue fractions of rhodopsin and *N*-retinylideneopsin, respectively, which are in a helical conformation. All of the usual reservations concerning the validity of this type of analysis of course apply (Gratzer, 1967; Rosenkranz, 1974). Using the average of the residue molar extinctions for the helix and random-coil forms of poly(glutamic acid) and polylysine at 190, 197, and 205 nm reported by Gratzer (1967) and assuming 324 residues per rhodopsin (this study), we estimate that 113 $\pm$ 6 (SE) residues undergo a transformation from the helical conformation, $\Delta f_H = 0.35 \pm 0.02$, following illumination under the specified experimental conditions. By itself this result implies a *minimum* helical content of about 35% for purified rhodopsin, prior to illumination. Circular dichroism measurements on purified rhodopsin indicate that about one-third of the rhodopsin helix is lost upon illumination (Shichi et al., 1969). The present result therefore is not inconsistent with a high rhodopsin helix content.

Oxidation of Amino Acid Residues during the Treatment of Rhodopsin with N-Bromosuccinimide. Since the spectral analysis of rhodopsin indicated the presence of two types of

Table III: Oxidation of Tryptophan, Histidine, and Tyrosine Residues of Rhodopsin with *N*-Bromosuccinimide

	mol/mol of rhodopsin		
time (min)	tryptophan	histidine	tyrosine
0	8	5	15
3	1.26	3.25	8.3
10	0.07	3.20	7.8
20	0.07	3.13	7.5
45	0.06	3.17	7.4

red-shifted tryptophan residues, we were interested to see whether tryptophan residues show varied reactivities toward chemical reagents. In a preliminary experiment, we incubated rhodopsin with 75-fold *N*-bromosuccinimide and followed the extent of destruction of tryptophan, histidine, and tryosine residues at appropriate intervals up to 45 min. The numbers of remaining residues determined with an amino acid analyzer and averaged for four samples for each reaction time are shown in Table III. Deviations between samples were no greater than 10% of the mean values. Destruction of two out of five histidines and seven out of fifteen tyrosines was complete in 3 min after the addition of the reagent. On the other hand, oxidation of tryptophan took a longer time. About 50% of the A_{498} absorption of rhodopsin was lost in 3 min of the reaction. About half of histidine as well as tyrosine residues which were not modified even after 45 min of reaction are probably buried inside the opsin molecule. Although all of the tryptophan residues were eventually destroyed by the chemical, it is interesting to note that one to two tryptophan residues seemed to be somewhat more resistant to the oxidizing agent. Further studies are required to correlate the reactivity and spectral properties of different classes of tryptophan residues.

Discussion

Liu & Chang (1971) reported that hydrolysis of proteins with toluene-4-sulfonic acid does not destroy tryptophan residues. Trayhurn et al. (1974) applied the reagent for hydrolysis of rod membranes and found 2.6 tryptophan residues per molecule of rhodopsin (molecular weight of rhodopsin was assumed to be 39 000). Simpson et al. (1976) described a modification of the method in which methanesulfonic acid was used as an acid catalyst. After hydrolysis of purified rhodopsin with this reagent, we determined 8 mol of tryptophan and 17 mol of tyrosine per mol of rhodopsin. Essentially identical results were obtained from the near-UV spectral analysis which assumed simple additivity of tryptophan absorbance and the presence of bathochromically shifted species. We believe that this close agreement, obtained by two independent analytical methods, firmly establishes the correct tryptophan content at eight per rhodopsin molecule. Ebrey & Honig (1975) have suggested that the aromatic residues of rhodopsin may be significantly hyperchromic in the UV. It is clearly unnecessary to invoke such a mechanism to account for the near-UV absorption of rhodopsin.

The near-UV spectral analysis of rhodopsin (Figure 2) indicates that the spectra of about half of the tyrosine and tryptophan residues are significantly red shifted. The result implies that half of these residues are shielded to some extent from the aqueous solvent and thus occupy relatively apolar sites in the protein. Shielding of tyrosine residues is explicitly confirmed by the *N*-bromosuccinimide oxidation experiment in which 7 of 15 residues survived extensive reaction. The *N*-bromosuccinimide experiment was less informative in regard to tryptophan environment. All tryptophans were eventually oxidized. However, one to two tryptophan residues appeared to be more resistant than the oxidizable tyrosines. This partial inconsistency between the spectral analysis and the *N*-bromosuccinimide experiment may in part be due to differences in the susceptibility of tyrosine and tryptophan to oxidation by *N*-bromosuccinimide. A second factor may be that retinal itself can be oxidized by *N*-bromosuccinimide (unpublished observations); thus, one or more tryptophans, previously shielded by retinal, may be exposed to the solvent (and *N*-bromosuccinimide) upon oxidation of the retinal chromophore by *N*-bromosuccinimide. The final result of *N*-bromosuccinimide incubation is complete bleaching of the rhodopsin chromophore in the dark. Regardless of the mechanism of this bleaching, loss of absorbance at 500 nm may be linked to oxidation of one or two buried tryptophans as suggested by Cooper & Hogen (1976) in a related study on the stability of rhodopsin toward *N*-bromosuccinimide.

One of the more notable results of the near-UV analysis is that two tryptophan residues occupy very apolar sites in unbleached rhodopsin (spectra shifted 10 nm). The light-induced difference spectrum (Figure 4) also requires the presence of highly red shifted species in the unbleached state, followed by the exposure of at least one of these residues to the aqueous solvent by the time *N*-retinylideneopsin is formed. Several lines of evidence also suggest the presence of highly red shifted tryptophan species in rhodopsin and additionally suggest that at least one residue is closely associated with the retinal chromophore. On the basis of linear dichroism measurements on magnetically oriented frog and cattle rods in the near-UV, Chabre & Breton (1979) have concluded that one tryptophan residue has an abnormally red shifted spectrum (shifted 9 nm) and is oriented so that the plane of the aromatic ring is normal to the membrane plane. They also concluded that this tryptophan residue undergoes an orientational change during the metarhodopsin I → metarhodopsin II transition. The results of our unpublished experiments on the linear dichroism of air-dried films of bovine rod membranes agree with those of Chabre & Breton (1979) concerning the initial orientation and spectral properties of tryptophan. UV absorption measurements by Rafferty (1979) on sonicated bovine rod membranes also reveal perturbation of aromatic residues; kinetic evidence was obtained which suggested that one tryptophan was exposed to an aqueous environment, coincident with the metarhodopsin I → metarhodopsin II transition. Ebrey (1972) observed light-induced changes in the intensity of fluorescence from suspensions of bovine rod membranes which he attributed to tryptophan. Since the spectral measurements cited above were made on rod membranes, the aromatic amino acid residue(s) affected by the photic bleaching of rhodopsin could have been attributed to a nonpigment protein. Present measurements of purified rhodopsin, however, establish unequivocally that the perturbation of aromatic residues previously observed on rod membranes is indeed associated with opsin.

The near-UV difference spectrum of purified bovine rhodopsin (Figures 1b and 3) is quite similar to the one measured by Rafferty (1979) for the metarhodopsin I → metarhodopsin II transition in sonicated rod membranes if displacements due to retinal chromophore absorption are considered. The difference spectrum obtained for sonicated membranes was interpreted as primarily arising from the perturbation of one tryptophan residue. However, the analysis given in this study suggests that several tyrosine residues are also perturbed, possibly by exposure to the aqueous solvent. Because of the similarity of the near-UV spectra, this result should be extended to the membrane case. However, light-induced changes in secondary protein structure are clearly not indicated in the

membrane, contrary to that observed for purified rhodopsin. The light-induced difference spectrum of sonicated rod membranes is relatively small in the far-UV and can be completely accounted for by the perturbation of a few aromatic residues, in particular by the exposure of one tryptophan (Rafferty, 1979).

In summary, the aromatic residue content of bovine rhodopsin is 8 tryptophans, 17 tyrosines, and 28 phenylalanines. In purified rhodopsin, approximately half of the tryptophan and tyrosine residues are shielded to some degree from the solvent. Two of the tryptophan residues occupy highly apolar environments in rhodopsin. Upon light-induced bleaching, at least one tryptophan residue and possibly four tyrosine residues are exposed to an aqueous environment. Most of these results are applicable to the membrane case.

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References

Albert, A., & Litman, B. D. (1978) *Biochemistry 17*, 3893–3900.

Blaustein, D. I., & Dewey, M. M. (1979) *J. Histochem. Cytochem. 27*, 788–793.

Chabre, M., & Breton, J. (1979) *Photochem. Photobiol. 30*, 295–299.

Collins, F. D., & Morton, R. A. (1950) *Biochem. J. 47*, 3–10.

Cooper, A., & Hogan, M. E. (1976) *Biochem. Biophys. Res. Commun. 68*, 178–182.

Donovan, J. W. (1969) in *Physical Principles and Techniques of Protein Chemistry* (Leach, S. J., Ed.) Part A, pp 101–169, Academic Press, New York.

Ebrey, T. G. (1972) *Photochem. Photobiol. 15*, 585–588.

Ebrey, T. G., & Honig, B. (1975) *Q. Rev. Biophys. 8*, 129–184.

Fairbanks, G., Steck, T. L., & Wallach, D. F. H. (1971) *Biochemistry 10*, 2606–2617.

Fukuda, M. N., Papermaster, D. S., & Hargrave, P. A. (1979) *J. Biol. Chem. 254*, 8201–8207.

Gratzer, W. B. (1967) in *Poly-α-amino Acids* (Fasman, G. D., Ed.) pp 177–238, Marcel Dekker, New York.

Heller, J. (1968) *Biochemistry 7*, 2906–2913.

Hirs, C. H. W. (1956) *J. Biol. Chem. 219*, 611–621.

Hong, K., & Hubbell, W. L. (1972) *Proc. Natl. Acad. Sci. U.S.A. 69*, 2617–2621.

Hubbard, R. (1969) *Nature (London) 221*, 435–437.

Kropf, A., Whittenberger, B. P., Goff, S. P., & Waggoner, A. S. (1973) *Exp. Eye Res. 17*, 591–606.

Liang, C.-J., Yamashita, K., Muellenberg, C. G., Shichi, H., & Kobata, A. (1979) *J. Biol. Chem. 254*, 6414–6418.

Liu, T.-Y., & Chang, Y. H. (1971) *J. Biol. Chem. 246*, 2842–2848.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951) *J. Biol. Chem. 193*, 265–275.

Marquardt, D. W. (1963) *J. Soc. Ind. Appl. Math. 11*, 431–441.

McDiarmid, R. (1965) Ph.D. Thesis, Harvard University, Cambridge, MA.

Rafferty, C. N. (1979) *Photochem. Photobiol. 29*, 109–120.

Rafferty, C. N., Cassim, J. Y., & McConnell, D. G. (1977) *Biophys. Struc. Mech. 2*, 277–320.

Ramachandran, L. K., & Witkop, B. (1967) *Methods Enzymol. 11*, 283–299.

Robinson, W. E., Gordon-Walker, A., & Bownds, D. (1972) *Nature (London) 235*, 112–114.

Rosenkranz, H. (1974) *Z. Klin. Chem. Klin. Biochem. 12*, 415–422.

Shichi, H. (1970) *Biochemistry 9*, 1973–1977.

Shichi, H., Lewis, M. S., Irreverre, F., & Stone, A. L. (1969) *J. Biol. Chem. 244*, 529–536.

Simpson, R. J., Neuberger, M. R., & Liu, T.-Y. (1976) *J. Biol. Chem. 251*, 1936–1940.

Trayhurn, P., Mandel, P., & Virmaux, N. (1974) *Exp. Eye Res. 19*, 259–265.

Tsunasawa, S., Narita, K., & Shichi, H. (1980) *Biochim. Biophys. Acta* (in press).

Wald, G., & Brown, P. K. (1953) *J. Gen. Physiol. 37*, 189–200.

Wetlaufer, D. B. (1962) *Adv. Protein Chem. 17*, 303–390.