# Thermodynamic Measurements of the Contributions of Helix-Connecting Loops and of Retinal to the Stability of Bacteriorhodopsin†

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ABSTRACT: Thermodynamic studies of bacteriorhodopsin (BR) have been undertaken in order to investigate the factors that stabilize the structure of a membrane protein. The stability of the native, intact protein was compared to that of protein with retinal removed, and/or cleaved in one or two of the loops connecting the transmembrane helices. The stability was assessed using differential scanning calorimetry and thermal denaturation curves obtained from ultraviolet circular dichroism and absorption spectroscopy. Retinal binding and the loop connections were each found to make a small contribution to stability, and even a sample that was cleaved twice as well as bleached to remove retinal denatured well above room temperature. Removal of retinal destabilized the protein more than cleaving once, and about as much as cleaving twice. Retinal binding and the connections in the loops were found to stabilize BR in independent ways. Cleavage of the molecule into fragments did not reduce the intermolecular cooperativity of the denaturation. Dilution of the protein by addition of excess lipid in order to eliminate the purple membrane crystal lattice also did not alter the cooperativity. These results are used to compare the relative importance of various contributors to the stability of BR.

Because parts of integral membrane proteins must fold in the hydrophobic region of lipid bilayers, the balance of interactions stabilizing the proteins' structure is likely to be both quantitatively and qualitatively different from that found in soluble proteins. The factors contributing to the stability of proteins that contain several transbilayer $\alpha$-helices can be separated conceptually into those stabilizing the helices themselves and those stabilizing the interactions between the helices in the tertiary structure (Popot & Engelman, 1990). It has been proposed that the stability of the helices in bilayers arises from main-chain hydrogen bonding and from the hydrophobic nature of the side chains (Engelman et al., 1986) and experiments have shown that some individual helices from a polytopic membrane protein can be considered to be independent folding domains (Kahn & Engelman, 1992; Hunt et al., 1991). The relative importance of the factors leading to the association of the helices has not been as thoroughly explored.

Possible major factors stabilizing helix–helix association in bacteriorhodopsin (BR)[1] can be divided roughly into four categories. First, the extramembranous loops connecting the helices may constrain the positions of the helices by being short or by forming secondary and tertiary structures. Second, polar amino acid side chains within the membrane may cause the helices to associate in such a way as to allow the formation of hydrogen bonds and ion pairs in order to minimize the exposure of polar groups to the hydrophobic fatty acyl chains of the lipids (Engelman, 1982). Third, the favorable packing of helices with each other as opposed to with lipids may drive the helices together in order to maximize van der Waals contacts and minimize the cavities that might result if the helices were each surrounded by lipids; lipids may associate with each other rather than with helices for similar reasons. Fourth, the binding interactions between the retinal moiety and the protein may stabilize the structure of the protein.

The experiments described here measure the extent to which cuts in two of the helix-connecting loops or removal of retinal reduces the stability of BR. It has been shown that cutting the first two loops does not prevent the first two helices, (A)[2] and (B), from associating properly with the fragment (CDEFG) containing the remaining five helices, even when these three fragments have been reconstituted into separate vesicles before they are allowed to interact (Kahn & Engelman, 1992). Also, covalent connections in the loop connecting (ABCDE) to (FG) (Liao et al., 1984; Sigrist et al., 1988) and in the loop connecting (AB) and (CDEFG) (Huang et al., 1981; Liao et al., 1983, 1984; Popot et al., 1987) have been shown not to be necessary for proper folding of the protein. These experiments establish that, for at least some of the loops, a covalent connection is not needed to bring the helices together and to keep them in their proper positions and orientations. Gilles-Gonzalez et al. (1991) have shown by genetic deletion that the loop between (AB) and (CDEFG) and the loop between (ABCDE) and (FG) can be greatly shortened without significantly affecting the function of BR. This means that the secondary or tertiary structures of these loops are not needed to specify the position and orientation of the helices to which they are connected. Further, R(AB-

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[1] Abbreviations: BR, bacteriorhodopsin; DKB, 3 mM potassium phosphate/150 mM KCl, pH 7; DSC, differential scanning calorimetry; KB, 30 mM potassium phosphate, 150 mM KCl, and 0.025% $NaN_3$, pH 6; KB/5, 6 mM potassium phosphate, 30 mM KCl, and 0.005% $NaN_3$, pH 6; NMR, nuclear magnetic resonance; SDS, sodium dodecyl sulfate; SDS buffer, 50 mM sodium phosphate, 5% SDS, and 0.025% $NaN_3$, pH 6, 7, or 8; UV-CD, ultraviolet circular dichroism.

[2] The nomenclature used is that presented in Kahn and Engelman (1992). The helices of BR are assigned letters; a dot between letters indicates a missing covalent connection between those helices; a prefixed R indicates the presence of retinal; the subscript indicates the state of the sample: sheets (s), vesicles (v), mixed micelles (m), detergent solubilized (d), dissolved in organic solvents (o).

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$CDEFG)_s$ can be cleaved at many locations in the loops by papain (Ovchinnikov et al., 1977; Abdulaev et al., 1978; Lee et al., 1987) or by proteinase K (Dumont et al., 1985) without affecting the integrity of the structure. The picture that emerges is that many of the loops are not essential to the process of bringing the helices together, or to holding them together correctly.

Retinal has been shown to contribute to the stability of BR since bleaching purple membrane by removing the retinal disrupts its two-dimensional crystallinity to some extent, possibly reflecting loss of long-range order (Hiraki et al., 1978). This means that some change is occurring in the structure. However, Popot et al. (1987) found that $(AB)_v$ and $(CDEFG)_v$ associate even in the absence of retinal based on the kinetics of the regeneration of BR, and Adair et al. (unpublished experiments) have used fluorescence energy transfer to demonstrate this association in the absence of retinal, indicating that retinal is not essential for BR to refold into something like its native structure.

The above observations do not allow the determination of the contributions of the loops or of retinal to the net stability of BR. Here, we assess the contributions of two of the loops and of retinal to the stability of BR by measuring the temperature and enthalpy of denaturation of BR in its native state and when cleaved in the loops and/or bleached to remove the retinal. Unfolding temperatures and enthalpies were measured by differential scanning calorimetry (DSC). van't Hoff enthalpies were calculated from melting curves obtained by ultraviolet circular dichroism spectroscopy (UV-CD) and by ultraviolet/visible absorption spectroscopy, as well as from the DSC traces. It is shown that retinal binding and covalent connections in each loop make modest contributions to the stability of BR. In addition, it is shown that the loops and retinal contribute to the stability in ways that are independent. Elimination of the crystal lattice present in purple membrane is shown not to alter the enthalpy or intermolecular cooperativity of the denaturation. Because the loops, the retinal, and the crystal lattice are not sufficient to explain the stability of BR, by implication side-by-side interactions between transmembranous helices must play a significant role in stabilizing the protein structure.

## MATERIALS AND METHODS

*Materials.* All aqueous solutions were made using water purified by a Milli-Q water system (Millipore). Baker PCS reagent-grade 90% formic acid was used; 200-proof ethanol was from Quantum Chemical Corp. *all-trans*-Retinal, ethanolamine, trypsin–chymotrypsin inhibitor (Bowman–Birk inhibitor), and TLCK-treated chymotrypsin were from Sigma. Retinal was dissolved in ethanol just before use, and the concentration was determined from the absorbance at 380 nm using an extinction coefficient of 42 800 $cm^{-1}$ $M^{-1}$. Sodium taurocholate was from Calbiochem. Electrophoresis-purity SDS was from Bio-Rad Laboratories.

*Buffers.* SDS Buffer: 50 mM sodium phosphate, 5% SDS, and 0.025% sodium azide, pH 6. KB: 30 mM potassium phosphate, 150 mM KCl, and 0.025% sodium azide, pH 6.0. KB/5: same as KB, but diluted 5-fold. DKB: 3 mM potassium phosphate/150 mM KCl, pH 7. Carbonate buffer: 50 mM potassium carbonate/150 mM KCl, pH 9.

*Scintillation Counting, Amino Acid Analysis, Preparation of Purple Membranes, Preparation of (AB) and (CDEFG), Preparation of Peptides (A) and (B), and Preparation of Halobacterium halobium Lipids.* All were performed as described in Popot et al. (1987), with the modifications given in Kahn and Engleman (1992). For ease of protein concentration determination, radioactively labeled purple membanes were also prepared by adding [$^3$H]leucine to defined growth medium as described in Popot et al. (1987). The labeled membranes were mixed with unlabeled membranes to give a specific activity of about 10–20 cpm/nmol of leucine. The specific activity was determined by scintillation counting and quantitative amino acid analysis. The protein concentration of samples made from the labeled material could then be easily measured by scintillation counting. (A) extends from residues 6 to 42 and (B) from 36 to 71. (A) and (B) overlap by seven residues, the overlap being entirely within the polar region of the sequence between the first and second helices. (A) ends within the second helix in the structure of Henderson et al. (1990), but before the beginning of the hydrophobic and therefore membrane-spanning region of that helix. (B) ends at the chymotryptic cleavage site used to make (AB) and (CDEFG). The overlap between (A) and (B) was included so as to give each of these extremely hydrophobic peptides a significant number of polar residues in order to promote solubility in mixed organic/aqueous solvent systems and in aqueous detergent solutions.

*Preparation of Samples for DSC. (a) Cleavage of BR.* For $(AB{\cdot}CDEFG)_s$ and $R(AB{\cdot}CDEFG)_s$ samples, purple membranes were bleached with hydroxylamine and cleaved with chymotrypsin as described in Popot et al. (1987), except that a chymotrypsin inhibitor was added after cleavage to prevent further degradation of the BR. After the membranes had been incubated with chymotrypsin for 3.5 h, the material was cooled on ice and diluted at least 1:1 with ice-cold water containing a 1:3 mass ratio of chymotrypsin–trypsin (Bowman–Birk) inhibitor to chymotrypsin present in the sample. After being pelleted, the membranes were resuspended in ice-cold water containing half as much inhibitor as before and were pelleted again. They were then resuspended in ice-cold water at 5–10 mg/mL, frozen, lyophilized, and stored at −20 °C. When resuspended in DKB or reconstituted into vesicles as described below and stored at 4° C, a silver-stained polyacrylamide gel showed that the samples underwent no further degradation by chymotrypsin even after 1 month, unlike material not treated with the chymotrypsin–trypsin inhibitor, which was extensively degraded after a few days.

*(b) Solubilization and Reconstitution of BR To Make DSC Samples. (i) $R(ABCDEFG)_v$.* Lyophilized purple membranes were dissolved in SDS buffer at about 1 mg/mL. The exact concentration was determined by scintillation counting. Purple membrane contains a 1:3 lipid to protein mass ratio. Native *Halobacterium halobium* lipids dissolved in SDS buffer were added to bring the lipid to protein mass ratio to 2:1. Taurocholate dissolved in SDS buffer was added at a 1:1 mass ratio with the protein. Retinal dissolved in ethanol was added to bring the total amount of retinal to 1.5 mol/mol of BR (purple membrane contains 1 mol/mol of protein); the final ethanol concentration did not exceed 0.5% by volume. The sample was then reconstituted by precipitating the dodecyl sulfate with potassium, as described in Popot et al. (1987).

*(ii) $(ABCDEFG)_v$.* Purple membranes were bleached, lyophilized, and dissolved in SDS buffer at about 1 mg/mL, and then reconstituted as described above except that retinal was not added.

*(iii) $R(AB{\cdot}CDEFG)_v$.* Purple membranes were bleached and cleaved as described above, lyophilized, dissolved in SDS buffer at about 1 mg/mL, and reconstituted with retinal as described above.

(*iv*) (*AB·CDEFG*)$_v$. Purple membranes were bleached and cleaved as described above, lyophilized, dissolved in SDS buffer at about 1 mg/mL, and reconstituted as described above except that retinal was not added.

(*v*) *R*(*A·B·CDEFG*)$_v$. Peptides (A) and (B) and (CDEFG) were prepared and transferred to SDS buffer as described by Kahn and Engelman (1992). They were mixed at a 2:2:1 molar ratio of (A)$_d$ to (B)$_d$ to (CDEFG)$_d$. Native lipids dissolved in SDS buffer were added at a 2:1 mass ratio of lipid to protein. Taurocholate dissolved in SDS buffer was added at a 1:1 mass ratio with the protein. Retinal dissolved in ethanol was added at a 1.5:1 molar ratio with (CDEFG); the final ethanol concentration did not exceed 0.5% by volume. The sample was then reconstituted as described by Popot et al. (1987).

(*c*) *Concentration of Samples.* After samples had been dialyzed for 3 days against KB to remove residual dodecyl sulfate, the vesicles were pelleted in a 60 Ti rotor (Beckman) at 60K rpm (255000*g* at $r_{avg}$) for 3 h. They were then resuspended in DKB (or cabonate buffer), brought to a volume of 2.2 mL, and sonicated until no particles remained (about 1 min). The samples were then dialyzed in Spectra/Por 2 tubing against about 200 volumes of DKB (or carbonate buffer) for 2 days with two changes per day at 4 °C. The samples were in the range of 3–5 mg/mL in protein (about 0.15 mM). They were stored at 4 °C and used as soon as possible.

*DSC.* Just before use, the samples were sonicated using a microtip probe at maximum power on a Heat Systems-Ultrasonics, Inc., sonifier. Three bursts were used for about 0.5 min each separated by about 1 min of cooling. The samples were immersed in room temperature water during sonication.

After sonication, samples were degassed with stirring in an evacuated chamber for 5–10 min at room temperature. Samples were immediately loaded into the calorimeter cell, and the final dialysis buffer (also degassed) was loaded into the reference cell. The cells were pressurized to about 40 psi with nitrogen.

The calorimeter was a MicroCal MC-2 scanning calorimeter (MicroCal, Inc.) connected to a PC's Limited Turbo PC computer. The cell volume was 1.2088 mL. Scans were run at 60 °C/h. A background scan collected with buffer in both cells within 1 day of each sample run was subtracted from each scan. The calibration of the excess specific heat was checked with electric pulses of known power.

*Preparation of Samples for Ultraviolet Circular Dichroism Measurements.* (*a*) *R*(*ABCDEFG*)$_v$ *and* (*ABCDEFG*)$_v$. R-(ABCDEFG)$_v$ and (ABCDEFG)$_v$ samples were reconstituted at a 2:1 lipid to protein mass ratio using the technique described above. The final molar ratio of retinal to BR in the R(ABCDEFG)$_v$ samples was 1.5:1.

(*b*) *Samples Reconstituted from Fragments.* (AB·CDEFG)$_v$ was reconstituted from chymotryptically prepared (AB) and (CDEFG) at a 2:1 lipid to protein mass ratio using the method described in Popot et al. (1987). A 1.5:1 molar ratio of (CDEFG)$_d$ to (AB)$_d$ was used. R-(AB·CDEFG)$_v$ was prepared by adding retinal to (AB·CDEFG)$_v$ at a molar ratio of 1.5:1 with (CDEFG). (A·B·CDEFG)$_v$ was prepared at a 2:1 lipid to protein mass ratio using the method described in Kahn and Engelman (1992). Chromophore-regenerated R(A·B·CDEFG)$_v$ was prepared by adding retinal at a 1.5:1 molar ratio with (CDEFG). (AB)$_v$, (CDEFG)$_v$, and (A·B)$_v$ were reconstituted without retinal at a 2:1 lipid to protein mass ratio using the method described in Kahn and Engelman (1992).

(*c*) *Final Preparation of Samples.* After reconstituted samples had been dialyzed against KB for 3 days, they were dialyzed against about 100 volumes of DKB (or carbonate buffer) for 2 days with two changes per day at 4 °C. They were stored at 4 °C. Just before use, samples were sonicated using a microtip probe at full power for six 10-s bursts separated by 1.5 min of cooling, with the samples submerged in room temperature water. Sonication reduces the size of the vesicles, thereby reducing light scattering during spectroscopic measurements. Samples were then degassed with stirring for 5–10 min in an evacuated chamber at room temperature, and were promptly loaded into the stoppered UV-CD cuvette. Sample concentrations were adjusted with DKB (or carbonate buffer) to give a UV-CD signal at 222 nm of approximately–40 mdeg at a temperature of 10 °C. The protein concentration was approximately 10 $\mu$M, or about 0.1–0.3 mg/mL.

*UV-CD Measurements.* A stoppered 1-mm path-length QS quartz cuvette (Hellma) was used in an Aviv Model 60DS circular dichroism spectropolarimeter. The temperature of the cuvette holder was regulated by a refrigerated circulating bath. A thermocouple probe (Bailey Instruments, Inc.) was inserted into the cuvette holder in order to monitor the temperature.

Scans were carried out at a data point interval of 1 nm, with an averaging time of 3 s and a bandwidth of 1.5 nm. A buffer scan run at each temperature point was subtracted from each sample scan. After the cell was brought to a given temperature, the sample was allowed to equilibrate for about 5 min before the spectrum was collected. The dynode voltage remained below 400 V, that is, within the linear range of the instrument, at wavelengths longer than 201 nm at 20 °C, and at wavelengths longer than 205 nm at 97 °C. For each sample, spectra were first collected at 10 °C intervals, with two spectra averaged at each temperature point. Then, on the basis of the apparent $T_m$ determined from these scans, new spectra were collected around the $T_m$ at a smaller interval, usually 3 °C, with three spectra averaged at each temperature point.

*Preparation of Samples for Absorption Spectroscopy Measurements.* Samples were prepared as for UV-CD measurements, as described above.

*Absorption Spectroscopy Measurements.* Stoppered 1-cm path-length semimicro QS quartz cuvettes (Hellma) were used in a Perkin Elmer Lambda 6 UV/vis spectrophotometer. The temperature of the cuvette holders was regulated by a circulating water bath. A thermocouple probe (Bailey Instruments, Inc.) was inserted into the sample cuvette holder in order to monitor the temperature.

Spectra were recorded at a scan rate of 300 nm/min, with a data point interval of 1 nm. The slit width was 1 nm, and the response time was 3 s. Degassed buffer was put in the reference beam. After the cells were brought to a given temperature, they were allowed to equilibrate for at least 5 min before a spectrum was collected. A base-line scan collected at room temperature with buffer in both cuvettes was subtracted from all scans. Peak heights were measured by drawing a straight line tangential to the tails of the peaks and measuring the distance from the line to the points in the peaks furthest from the line. This method underestimates the peak heights to some extent.

*X-ray Diffraction Measurements.* Between 0.5 and 1 mg of protein, either in sheets or reconstituted into vesicles, was pelleted in an SW60 rotor at 60K rpm (370000*g* at $r_{avg}$) for 70 min, usually from KB. The pellet was resuspended in KB/5 and pelleted again, the supernatant carefully removed with

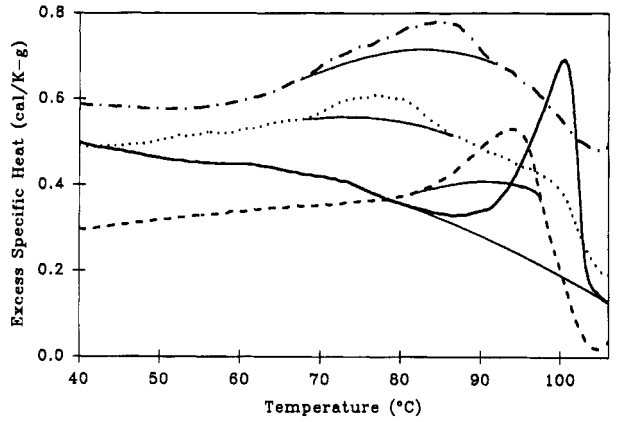


FIGURE 1: DSC traces of membrane sheets in DKB: R(ABCDEFG)$_s$ (—); R(AB·CDEFG)$_s$ (- - -); (ABCDEFG)$_s$ (– · –); (AB·CDEFG)$_s$ (···). The vertical positions have been shifted arbitrarily for clearer presentation. The base lines for the traces are shown as solid lines.

a drawn-out Pasteur pipet, and the inside of the tube dried with a tissue. One to two microliters of KB/5 was added back to the pellet, which was homogenized with a stir bar (1 × 2 mm) for about 5 min. The consistency of the sample was as thick as possible, but without any lumps. The tube was then spun briefly in a table-top centrifuge to coalesce the sample. The sample was transferred to a 1.5-mm-diameter glass X-ray capillary (Charles Supper Co., Inc.). The capillary was placed in a holder and spun in a table-top swinging-bucket centrifuge ("Dynac", Adams) at maximum speed for about 1 min to pack the sample in the bottom of the capillary. The capillary was then sealed with melted wax. X-ray diffraction patterns were collected and analyzed as described in Kahn and Engelman (1992). The sample-to-film distance was 7.2 cm.

## RESULTS

### *Calorimetry*

*Membrane Sheets.* Samples were prepared of R(ABCDEFG)$_s$, (ABCDEFG)$_s$, R(AB·CDEFG)$_s$, and (AB·CDEFG)$_s$ in DKB. The pH used was a compromise between reducing the temperature of denaturation in order to facilitate the experiments, and maintaining the proper absorption spectrum of the samples. Samples reconstituted from fragments at a higher lipid to protein ratio than that in sheets (see below) lose their native absorption spectra at a pH higher than 7 (Kahn & Engelman, 1992). DSC of sheets was also performed at pH 9 in carbonate buffer, but these results cannot be compared directly to the spectroscopic measurements that were made at pH 7.

The excess specific heat curves are shown in Figure 1. Base lines were drawn in by hand as shown in the figure, and the areas were measured with a planimeter to give the calorimetric enthalpies. In the case of R(AB·CDEFG)$_s$, the large exothermic event that occurs at high temperature in all of the samples overlaps to some extent with the denaturation peak, making it difficult to draw the base line. This exothermic event may be the aggregation of denatured BR molecules [as also suggested by Brouillette et al. (1987)]. Because the base lines are not flat, we were unable to determine the value of $\Delta C_p$. This may add some uncertainty to the $\Delta H$ values, but does not change the qualitative conclusions presented below.

Table I: DSC Results

| sample | $T_m$ (°C) | $\Delta H_{cal}$ (kcal/mol) | $\Delta H_{vH}$ (kcal/mol) | $\Delta H_{vH}/\Delta H_{cal}$ |
|---|---|---|---|---|
| R(ABCDEFG)$_s$ | 101 | 100 | 148 | 1.5 |
| R(AB·CDEFG)$_s$ | 95 | 56 | 110 | 2.0 |
| (ABCDEFG)$_s$ | 85 | 23 | 76 | 3.3 |
| (AB·CDEFG)$_s$ | 80 | 16 | 84 | 5.4 |
| R(ABCDEFG)$_s$, pH 9 | 83 | 148 | 111 | 0.75 |
| R(AB·CDEFG)$_s$, pH 9 | 78 | 62 | 127 | 2.1 |
| (ABCDEFG)$_s$, pH 9 | 65 | 56 | 71 | 1.3 |
| (AB·CDEFG)$_s$, pH 9 | 57 | 22 | 80 | 3.7 |
| R(ABCDEFG)$_v$ | 95 | 121 | 191 | 1.6 |
| R(AB·CDEFG)$_v$ | 83 | 88 | 94 | 1.1 |
| (ABCDEFG)$_v$ | 79 | 20 | 122 | 6.1 |
| (AB·CDEFG)$_v$ | 71 | 20 | 56 | 2.8 |
| R(A·B·CDEFG)$_v$ | 71 | 39 | 67 | 1.7 |

Table II: Relative Results from DSC of Membrane Sheets

| sample | $\Delta T_m$ (°C) | $\Delta\Delta H_{cal}$ (kcal/mol) | $\Delta\Delta H_{vH}$ (kcal/mol) |
|---|---|---|---|
| R(ABCDEFG)$_s$ | 0 | 0 | 0 |
| R(AB·CDEFG)$_s$ | –6 | –44 | –38 |
| (ABCDEFG)$_s$ | –16 | –77 | –72 |
| (AB·CDEFG)$_s$ | –21 | –84 | –64 |

It is also possible to determine the van't Hoff enthalpy from the DSC data using the formula:

$$\Delta H_{vH} = 4RT_{1/2}^2 \frac{C_p(T_{1/2})}{\Delta H(T_{1/2})}$$

where $R$ is the gas constant, $T_{1/2}$ is the temperature at which the transition is half complete (for the purposes of this paper, taken to be the maximum of the peak), $C_p(T_{1/2})$ is the heat capacity at $T_{1/2}$ (taken to be the distance from the base line to the top of the peak), and $\Delta H(T_{1/2})$ is the enthalpy evaluated at $T_{1/2}$ (taken to be the area under the peak) (Sturtevant, 1987). A comparison of the van't Hoff enthalpy with the calorimetric enthalpy can give an indication of the extent of intermolecular cooperativity in the transition.

Table I shows the results for these four samples. The table gives the $T_m$, which corresponds to the point of maximal excess heat measured from the base line to the top of the peak, $\Delta H_{cal}$ measured from the area of the peak, and $\Delta H_{vH}$ determined from the DSC data. The $T_m$ can be determined to within about 1 °C. The $\Delta H_{cal}$ and $\Delta H_{vH}$ values can be determined to within about ±10% for the purple membrane samples which have large, well-defined peaks, but the error increases for the other samples, reaching about ±50% for the samples with the smallest peaks because of difficulties in deciding where to draw the base lines (see also the samples reconstituted into vesicles, below). The $\Delta H_{vH}$ values are probably the least certain since the calculation assumes a symmetric peak, and the peaks obtained in these experiments are asymmetric and frequently have shoulders as well.

Table II shows how the thermodynamic values for the cleaved and bleached samples compare to those of R(ABCDEFG)$_s$. As shown by the changes in $T_m$, bleaching either intact or cleaved material reduces the stability more than cleaving either native or bleached material. Bleaching either intact or cleaved material reduces the $T_m$ by about the same, large, amount, and cleaving either native or bleached material reduces the $T_m$ by about the same, small, amount. Likewise, the changes in enthalpy show that bleaching the protein has a greater effect than cleaving it. Cleavage of material that has been bleached results in less destabilization than cleavage of R(ABCDEFG)$_s$. It is also the case, however, that bleaching

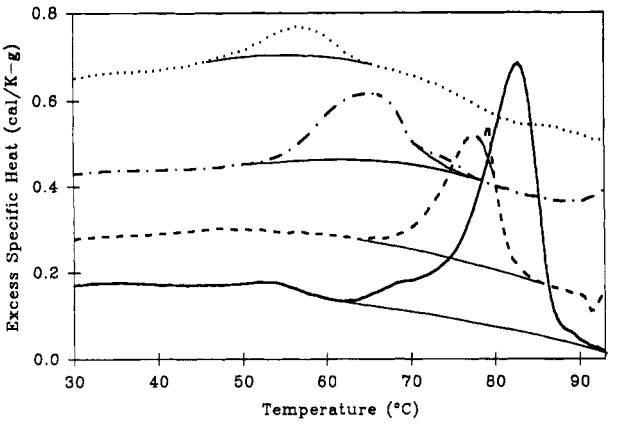


FIGURE 2: DSC traces of membrane sheets at pH 9: R(ABCDEFG)$_s$ (—); R(AB·CDEFG)$_s$ (- - -); (ABCDEFG)$_s$ (– · –); (AB·CDEFG)$_s$ (···). The vertical positions have been shifted arbitrarily for clearer presentation. The base lines for the traces are shown as solid lines.

Table III: Relative Results from DSC of Membrane Sheets at pH 9

| sample | $\Delta T_m$ (°C) | $\Delta\Delta H_{cal}$ (kcal/mol) | $\Delta\Delta H_{vH}$ (kcal/mol) |
|---|---|---|---|
| R(ABCDEFG)$_s$, pH 9 | 0 | 0 | 0 |
| R(AB·CDEFG)$_s$, pH 9 | –5 | –86 | +16 |
| (ABCDEFG)$_s$, pH 9 | –18 | –92 | –40 |
| (AB·CDEFG)$_s$, pH 9 | –26 | –126 | –31 |

cleaved material does not decrease the $\Delta H_{cal}$ by as much as bleaching intact material. Thus, in summary, the binding of retinal contributes more to the stability of BR than does the covalent connection in the loop, and although bleaching influences the effect of cleaving and vice versa, there is some degree of independence between these two stabilizing factors. In fact, the changes in $T_m$ are quite independent. Cleaving the molecule does not abolish the contribution of retinal to the stability, nor does bleaching abolish the contribution of the covalent connection in the loop.

$\Delta S$ can be calculated from the formula $\Delta G = \Delta H - T\Delta S$, where $T$ is $T_{1/2}$ (taken to be $T_m$, the maximum of the peak), so that $\Delta G$ is zero. The effect of cleaving the B–C loop on the stability of the protein is both entropic and enthalpic (calculation not shown).

The ratio of the van't Hoff enthalpy to the calorimetric enthalpy can give a measure of the extent of intermolecular cooperativity. Table I shows that this value is greater than 1 for each of the samples, indicating that there is some intermolecular cooperativity.

The values for $T_m$, $\Delta H_{cal}$, and extent of intermolecular cooperativity reported here for R(ABCDEFG)$_s$ are similar to those reported by Jackson and Sturtevant (1978) and Brouillette et al. (1987).

DSC was also performed on R(ABCDEFG)$_s$,R(AB·CDEFG)$_s$, (ABCDEFG)$_s$, and (AB·CDEFG)$_s$ at pH 9 in carbonate buffer, and the traces and base lines are shown in Figure 2. As explained earlier, the results of these experiments cannot be compared directly to the spectroscopic results described below because (AB) + (CDEFG) + R and (A) + (B) + (CDEFG) + R do not regenerate properly at pH 9 at a 2:1 lipid to protein mass ratio. The results of the DSC at pH 9 are shown in Table I. Table III shows how bleached and cleaved samples compare to intact material. The effects of bleaching, cleaving, and both on $T_m$ and $\Delta H_{cal}$ are similar to the effects seen at pH 7. Although the $\Delta\Delta H_{cal}$ values for the samples at pH 9 are similar to those at pH 7, the absolute values at pH 9 are higher. This is unexpected since Brouillette et al. (1987) found that in R(ABCDEFG)$_s$, the higher the pH, the lower the enthalpy of denaturation. It is possible that the use of carbonate buffer instead of phosphate affected the enthalpies; Brouillette et al. found that the enthalpy for R(ABCDEFG)$_s$ is dependent on the choice of buffer as well as the pH.

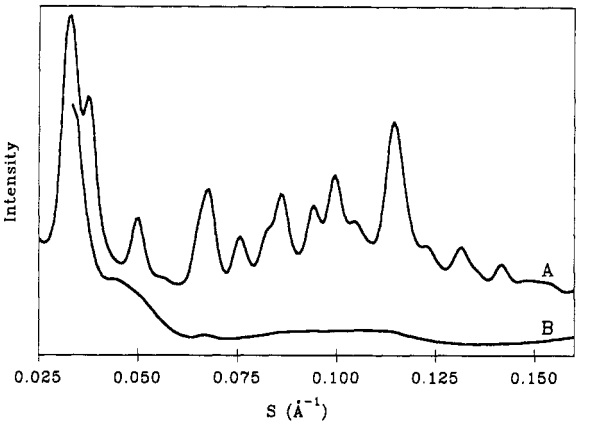


FIGURE 3: Radial averages of X-ray diffraction patterns of wet pellets: (A) R(ABCDEFG)$_s$; (B) R(ABCDEFG)$_v$.

*Vesicles.* In membrane sheets, the lipid to protein mass ratio is only 1:3, and in the case of R(ABCDEFG)$_s$ and R(AB·CDEFG)$_s$, the protein is present in the form of a two-dimensional crystal lattice. This very low lipid to protein ratio forces the protein molecules to be very close together, and the resulting interactions between protein molecules may contribute to the stability of the native structure. In order to assess the contribution of the tight packing of the molecules to the stability of the samples, the DSC experiments were repeated using samples that were reconstituted into vesicles at a 2:1 lipid to protein mass ratio using native *H. halobium* lipids. This lipid to protein ratio was chosen because it was the highest ratio at which (A) + (B) + (CDEFG) + R would regenerate properly (Kahn & Engelman, 1992). In a sample consisting of 100%-regenerated BR, a 2:1 mass ratio of lipid to protein would correspond approximately to a 60:1 molar ratio.

X-ray diffraction of a wet pellet of R(ABCDEFG)$_v$ was used to check whether the crystal lattice had been eliminated. No lattice was detected; by comparison, when a wet pellet of R(ABCDEFG)$_s$ is examined, a lattice is recorded on the film (see Figure 3). Thus, in the reconstituted samples, the protein molecules are not constrained by a crystal lattice. It is possible that in the reconstituted samples the BR molecules exist as trimers, since visible UV-CD experiments have shown that in DMPC vesicles at this lipid to protein ratio, exciton coupling can be observed (Cherry et al., 1978) [although Du et al. (1990) have evidence that the exciton coupling may not be due to the trimeric arrangement of BR]. Also, Hiraki et al. (1981) have calculated the expected diffraction pattern from trimeric, noncrystalline BR based on a simple model of the molecule. The calculated pattern is very similar to the one obtained here from a wet pellet of R(ABCDEFG)$_v$. Even though the BR in these samples may be trimeric, the absence of a crystal lattice might be expected to eliminate a restriction on the unfolding of the molecule. Comparison of DSC measurements of samples in the form of sheets with samples in the form of vesicles can give a measurement of the magnitude of this effect.

Figure 4 shows the calorimeter traces and base lines for the reconstituted samples. For each sample, the excess specific heat was determined by dividing the excess heat by the amount of properly regenerated BR in each sample. The extent of

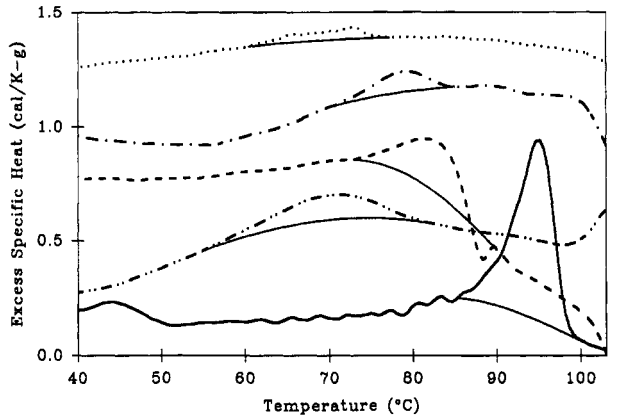


FIGURE 4: DSC traces of samples reconstituted in vesicles: R(AB-CDEFG)$_v$ (—); R(AB·CDEFG)$_v$ (- - -); (ABCDEFG)$_v$ (— · —); (AB·CDEFG)$_v$ (···); R(A·B·CDEFG)$_v$ (— · · —). The vertical positions have been shifted arbitrarily for clearer presentation. The base lines for the traces are shown as solid lines.

regeneration was determined by measuring the concentration of total protein in each sample by scintillation counting of the tritiated leucines contained in the protein, and comparing the concentration to the absorbance of the chromophore, using an extinction coefficient of 45 000 cm$^{-1}$ M$^{-1}$, the same as that of R(AB·CDEFG)$_v$ (Popot et al., 1987). The R(ABCDEFG)$_v$ and R(AB·CDEFG)$_v$ samples were found to be approximately 60% regenerated. The remaining 40% of the material was assumed to be in a permanently denatured state which should not unfold cooperatively at the same temperature as the properly regenerated material. Since the (ABCDEFG)$_v$ sample differs from the R(ABCDEFG)$_v$ sample and the (AB·CDEFG)$_v$ sample differs from the R(AB·CDEFG)$_v$ sample only in that retinal was not added, it was assumed that the extent of proper reconstitution was the same for those samples, respectively. Actually, the (ABCDEFG)$_v$ and (AB·CDEFG)$_v$ samples both contained some residual retinal-containing material that probably had not been cleaved by chymotrypsin. This amounted to only about 6% of each sample, so its presence was not considered to be significant. Furthermore, it probably denatures at a significantly higher temperature than (ABCDEFG)$_v$ or (AB·CDEFG)$_v$, and so should not add area to the transition peaks. The R(A·B·CDEFG)$_v$ sample was approximately 28% regenerated. Because of the extremely low enthalpy measured for the (AB·CDEFG)$_v$ sample, DSC measurements were not done on (A·B·CDEFG)$_v$ (although UV-CD measurements were done; see below).

Table I shows the values obtained from the DSC traces. For R(ABCDEFG)$_v$, R(AB·CDEFG)$_v$, (ABCDEFG)$_v$, and (AB·CDEFG)$_v$, it can be seen that the calorimetric enthalpies are similar to those of the corresponding samples in the form of sheets. The ratios of van't Hoff enthalpies to calorimetric enthalpies are also above 1. Thus, it appears that elimination of the crystal lattice does not affect the enthalpy of denaturation and does not eliminate intermolecular cooperativity. The only effect is to lower the denaturation temperature. Thus, the lattice stabilizes BR with respect to temperature, but does not otherwise affect the unfolding process; that is, the effect is entropic and not enthalpic.

The relative values of the DSC results for the reconstituted samples are given in Table IV. The effect on the $T_m$ of cleaving once is somewhat greater than in sheets. The effect of bleaching or bleaching plus cleaving once is similar to that in sheets. The effect of two breaks in the sequence is the same as the effect of bleaching plus cleaving once. The changes in

Table IV: Relative Results from DSC of Vesicles

| sample | $\Delta T_m$ (°C) | $\Delta\Delta H_{cal}$ (kcal/mol) | $\Delta\Delta H_{vH}$ (kcal/mol) |
|---|---|---|---|
| R(ABCDEFG)$_v$ | 0 | 0 | 0 |
| R(AB·CDEFG)$_v$ | −12 | −33 | −97 |
| (ABCDEFG)$_v$ | −16 | −101 | −69 |
| (AB·CDEFG)$_v$ | −24 | −101 | −135 |
| R(A·B·CDEFG)$_v$ | −24 | −82 | −124 |

enthalpy in vesicles are also similar to those in sheets. However, the enthalpy for (ABCDEFG)$_v$ and (AB·CDEFG)$_v$ was the same, which would indicate that cleaving (ABCDEFG)$_v$ does not reduce the enthalpy at all. However, because of the very small enthalpies measured for (ABCDEFG)$_v$ and particularly for (AB·CDEFG)$_v$, this may simply be due to errors in the measurement.

R(A·B·CDEFG)$_v$ has a denaturation enthalpy that is greater than that of (ABCDEFG)$_s$, (AB·CDEFG)$_s$, (ABCDEFG)$_v$, or (AB·CDEFG)$_v$, but less than that of R(AB·CDEFG)$_s$ or R(AB·CDEFG)$_v$. This means that the enthalpic effect of removing the retinal is greater than that of cutting two of the loops connecting the helices, although the effect on $T_m$ is less. The effects on the $T_m$ and calorimetric enthalpy of removing the second connection are roughly comparable to the effects of removing the first.

### *Spectroscopic Measurements*

The van't Hoff enthalpy of denaturation can be determined from any measurable parameter of a protein that changes cooperatively upon denaturation. The van't Hoff enthalpy is determined from the change in the equilibrium constant with temperature. No information is needed about the amount of protein in the sample or the molecular weight of the protein.

*Ultraviolet Circular Dichroism.* The UV-CD spectrum of BR is indicative of a largely helical structure (Mao & Wallace, 1984; Wallace & Teeters, 1987). To a first approximation, the ellipticity at 222 nm is a measure of the helical content of the sample. If BR is heated, the ellipticity at 222 nm decreases in a cooperative way, indicating that a denaturation event can be monitored by this method. The loss of ellipticity probably corresponds to a loss of helicity, but for the purposes of studying the thermodynamics of the denaturation, it is not necessary to know exactly what structural changes are occurring.

All samples were reconstituted into vesicles at a 2:1 lipid to protein mass ratio with native lipids. Data could not be collected on R(ABCDEFG)$_v$ at pH 7 because the denaturation temperature is too high. However, spectra were recorded for R(ABCDEFG)$_v$ at pH 9 in carbonate buffer, and these results can be compared to the DSC of R(ABCDEFG)$_v$ at pH 9.

Figure 5a shows the background-subtracted spectra for R(AB·CDEFG)$_v$. Below the transition, the spectra have a typical $\alpha$-helical appearance. As the sample is heated, the ellipticity decreases, possibly because of an increase in light scattering or a gradual small change in conformation. As the temperature continues to rise, the ellipticity passes through a transition; that is, there is a cooperative loss of ellipticity. The ellipticity then goes back to decreasing at a gradual rate. Above the transition, the spectra no longer have the double minima characteristic of $\alpha$-helices, but the minimum at about 222 nm remains, indicating that a substantial amount of helix is still present. Brouillette et al. (1987) observed the same shift from a double to a single minimum in their UV-CD spectra of R(ABCDEFG)$_s$, and suggested that the change in shape might be due to an increase in light scattering.

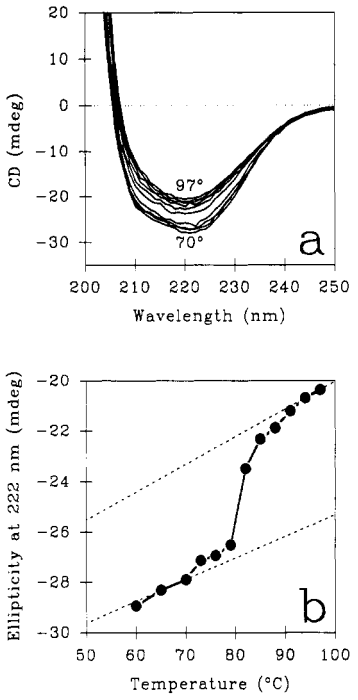


FIGURE 5: (a) UV-CD spectra of R(AB·CDEFG)$_v$ at 3 °C intervals from 70 to 97 °C. (b) Ellipticity at 222 nm versus temperature of R(AB·CDEFG)$_v$. The dashed lines show the base lines used in determining the equilibrium constant at each data point.

Figure 5b shows a plot of the ellipticity at 222 nm as a function of temperature. The cooperative nature of the transition is apparent from the sigmoidal shape of the curve. Base lines have been drawn through the slopes of the data before and after the transition. These slopes represent the rate at which ellipticity was lost as a result of increasing light scattering or small conformational changes with temperature.

To determine the van't Hoff enthalpy for the transition, the equilibrium constant at each temperature was calculated. Before the transition, the protein was considered to be entirely in the native state, and after the transition to be entirely in the denatured state. The equilibrium constant was taken to be the distance from a data point to the lower base line divided by the distance from that data point to the upper base line.

The van't Hoff plots for all samples are shown in Figure 6. Table V gives the thermodynamic values. The midpoint temperature was determined by using interpolation to find the point at which ln $K$ is equal to zero. The $T_m$ values can be determined to within about ±5 °C, and the $\Delta H_{vH}$ values can be determined to within about ±50%. (AB)$_v$ and (A·B)$_v$ showed no cooperative transitions at the sensitivity of these experiments. (A·CDEFG)$_v$, (B·CDEFG)$_v$, and (CDEFG)$_v$ showed cooperative transitions that were too small to measure accurately.

Table VI shows the relative values for the UV-CD experiments. The changes in $T_m$ and $\Delta H_{vH}$ relative to R(ABCDEFG)$_v$ at pH 9 are underestimated because the protein is less stable at pH 9 than at pH 7. The $T_m$ values are similar to those obtained by DSC of vesicle samples, except for (ABCDEFG)$_v$. The determination of the $T_m$ by this method is not as precise as by DSC. Bleaching R(A·B·CDEFG)$_v$ reduces its $T_m$ (this experiment was not performed by DSC). The effect on the $T_m$ of the second cleavage is greater than the effect of the first cleavage when retinal is present, though not when it is absent. As with DSC of sheets, bleaching reduces the enthalpy by more than cleaving once, and the effects of bleaching and of cleaving appear to be somewhat independent. These results are also similar to the results of the DSC experiments on samples in vesicles except for the fact that DSC indicated that (ABCDEFG)$_v$ and (AB·CDEFG)$_v$ had the same enthalpy of denaturation. As proposed above, this may be due to errors arising from the very small peaks obtained in the DSC experiments. Unlike the DSC experiments, the UV-CD experiments indicate that removing the retinal from R(ABCDEFG)$_v$ does not have as great an effect on the enthalpy as cutting two of the loops, although this may be due to the fact that the $\Delta H_{vH}$ for R(ABCDEFG)$_v$ was measured at pH 9, as mentioned above. The enthalpy of (A·B·CDEFG)$_v$ is only slightly less than the enthalpy of R(A·B·CDEFG)$_v$. The enthalpy of (A·B·CDEFG)$_v$ is only slightly greater than that of (AB·CDEFG)$_v$. This presumably reflects the errors present in the figures.

A curious result is that the van't Hoff enthalpies derived from the UV-CD results are closer in value to the calorimetric enthalpies than to the van't Hoff enthalpies derived from the DSC data. If a direct comparison can be made between the UV-CD results and the DSC results, this would indicate that the cooperative unit is one molecule, rather than more than one as indicated by a comparison of the van't Hoff enthalpies derived from the DSC data to the calorimetric enthalpies. The presence of an irreversible step in the denaturation at a temperature somewhat above the main denaturation temperature can reduce the measured enthalpy, and since the UV-CD experiments take much longer than the DSC experiments, there is more time for the irreversible step to occur (see Discussion). Similar results have been obtained for ribonuclease $T_1$, with the $\Delta H_{vH}$ determined from thermal denaturation curves agreeing well with the calorimetric $\Delta H$, despite the fact that the $\Delta H_{vH}$ determined from DSC traces was greater than the calorimetric $\Delta H$ (Hu et al., 1992).

*Absorption Spectroscopy.* When retinal is bound to BR, its absorbance maximum is shifted from 380 nm to about 560 nm. As a sample of BR is heated, the bound retinal peak shrinks, and the free retinal peak grows in a cooperative way. This means that a van't Hoff enthalpy for the loss of retinal can be calculated on the basis of the change in the equilibrium constant with temperature. This type of experiment was performed on samples of R(ABCDEFG)$_v$, R(AB·CDEFG)$_v$, and R(A·B·CDEFG)$_v$ reconstituted in native lipid vesicles at a 2:1 lipid to protein mass ratio under the same conditions as for the UV-CD experiments.

As R(ABCDEFG)$_v$ is heated, at first the absorbance maximum shifts toward the blue and decreases in a noncooperative way (data not shown). At higher temperatures, there is a cooperative loss of the bound chromophore and a simultaneous increase in the free retinal chromophore. The range of 84–93 °C shows a cooperative loss of the bound chromophore absorbance at about 500 nm and a simultaneous increase in the free retinal absorbance at 380 nm, with an isosbestic point at 412 nm (Figure 7a). It is in this range that the retinal is being lost cooperatively from the protein, and a van't Hoff enthalpy for this process can be calculated. The presence of an isosbestic point shows that the loss of retinal is probably a two-state process.

84 °C was taken to be the temperature at which the material was entirely in the native state, and 93 °C was taken to be the temperature at which no bound retinal was left. The equilibrium constant at a temperature point was taken to be the difference in absorbance between that point and 84 °C, divided by the difference in absorbance between that point and 93 °C. Figure 8 shows the van't Hoff plot.

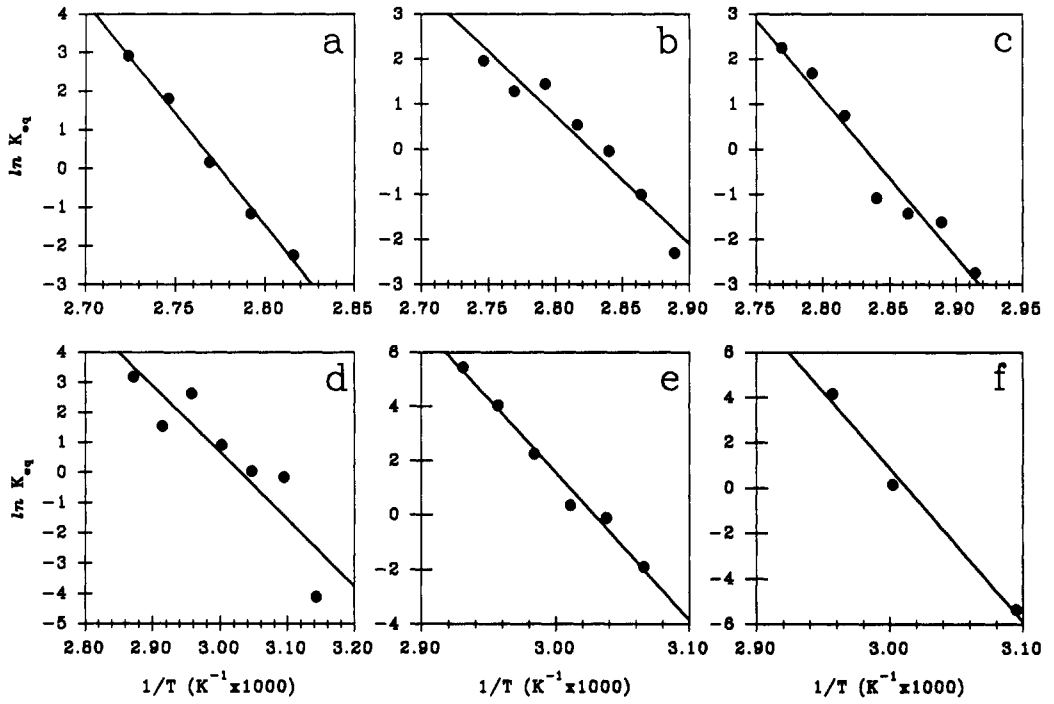


FIGURE 6: van't Hoff plots based on ellipticity at 222 nm of samples reconstituted in vesicles. The lines of best fit were determined by linear regression. (a) R(ABCDEFG)$_v$ at pH 9; (b) (ABCDEFG)$_v$; (c) R(AB·CDEFG)$_v$; (d) (AB·CDEFG)$_v$; (e) R(A·B·CDEFG)$_v$; (f) (A·B·CDEFG)$_v$.

Table V: UV-CD Results

| sample | $T_m$ (°C) | $\Delta H_{vH}$ (kcal/mol) | % of $\theta_{222}$ lost |
|---|---|---|---|
| R(ABCDEFG)$_v$, pH 9 | 87 | 115 | 34 |
| R(AB·CDEFG)$_v$ | 80 | 70 | 25 |
| (ABCDEFG)$_v$ | 81 | 57 | 31 |
| (AB·CDEFG)$_v$ | 59 | 26 | 23 |
| R(A·B·CDEFG)$_v$ | 63 | 34 | 26 |
| (A·B·CDEFG)$_v$ | 53 | 30 | 18 |

Table VI: Relative Results from UV-CD

| sample | $\Delta T_m$ (°C) | $\Delta\Delta H_{vH}$ (kcal/mol) |
|---|---|---|
| R(ABCDEFG)$_v$, pH 9 | 0 | 0 |
| R(AB·CDEFG)$_v$ | −7 | −45 |
| (ABCDEFG)$_v$ | −6 | −58 |
| (AB·CDEFG)$_v$ | −28 | −89 |
| R(A·B·CDEFG)$_v$ | −24 | −81 |
| (A·B·CDEFG)$_v$ | −34 | −85 |

The spectra of R(AB·CDEFG)$_v$ and R(A·B·CDEFG)$_v$ in vesicles were analyzed in the same way. As R(AB·CDEFG)$_v$ is heated, first there is a gradual, noncooperative shift in the position and magnitude of the absorbance (data not shown). Then, the range from 65 to 90 °C shows a cooperative loss of the bound chromophore and a simultaneous increase in the unbound chromophore, with an isosbestic point at about 420 nm (Figure 7b). The isosbestic point has been blurred somewhat, perhaps because as the temperature is increased the light scattering of the sample increases, as was the case in the UV-CD experiments; 65 °C was taken to be the temperature at which the retinal was entirely bound, and 90 °C was taken to be the temperature at which the retinal was entirely unbound. Figure 8 shows the van't Hoff plot.

Figure 7c shows the spectra over the range of 30–70 °C for R(A·B·CDEFG)$_v$. It can be seen that there are two peaks, one at about 550 nm and one at about 495 nm, which both decrease cooperatively at about the same rate as the peak at 380 nm increases. Using low-temperature spectroscopy, Kataoka et al. (1992) also have found that there are two species

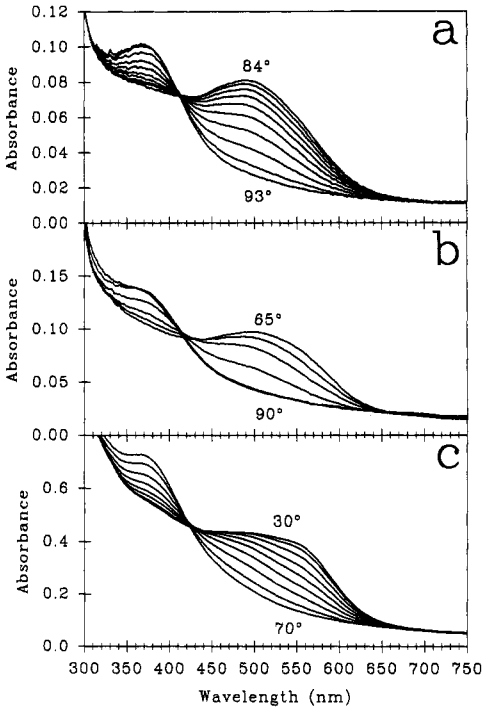


FIGURE 7: (a) Absorption spectra of R(ABCDEFG)$_v$ at 1 °C intervals from 84 to 93 °C. (b) Absorption spectra of R(AB·CDEFG)$_v$ at 5 °C intervals from 65 to 90 °C. (c) Absorption spectra of R(A·B·CDEFG)$_v$ at 5 °C intervals from 30 to 70 °C.

at equilibrium in this sample, both of which undergo photocycles, and therefore presumably are functional. Pande et al. (1989) found that subtle changes in the environment of the retinal can shift the absorption maximum to the vicinity of 480 nm without significant change in the secondary structure of the protein. Upon heating the sample, an isosbestic point is seen at about 425 nm; 30 °C was taken to be the temperature at which the retinal was completely bound and 70 °C the temperature at which the retinal was completely unbound. Figure 8 shows the van't Hoff plot.

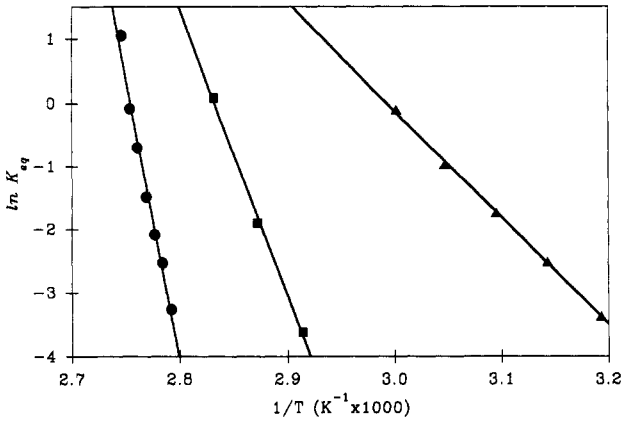


FIGURE 8: van't Hoff plots based on absorbance at 380 nm of R(ABCDEFG)$_v$ (●), R(AB·CDEFG)$_v$ (■), and R(A·B·CDEFG)$_v$ (▲). The lines of best fit were determined by linear regression.

Table VII: Absorption Spectroscopy Results

| sample | $T_m$ (°C) | $\Delta H_{vH}$ (kcal/mol) |
|---|---|---|
| R(ABCDEFG)$_v$ | 90 | 179 |
| R(AB·CDEFG)$_v$ | 80 | 90 |
| R(A·B·CDEFG)$_v$ | 60 | 34 |

Table VIII: Relative Results from Absorption Spectroscopy

| sample | $\Delta T_m$ (°C) | $\Delta\Delta H_{vH}$ (kcal/mol) |
|---|---|---|
| R(ABCDEFG)$_v$ | 0 | 0 |
| R(AB·CDEFG)$_v$ | −10 | −89 |
| R(A·B·CDEFG)$_v$ | −30 | −145 |

Table IX: Comparisons of Calorimetric and van't Hoff Enthalpies

| sample | $\Delta H_{vH}$(UV-CD)/$\Delta H_{cal}$ | $\Delta H_{vH}$(ABS)/$\Delta H_{cal}$ |
|---|---|---|
| R(ABCDEFG)$_v$ | 0.95 | 1.50 |
| R(AB·CDEFG)$_v$ | 0.79 | 1.02 |
| R(A·B·CDEFG)$_v$ | 0.88 | 0.87 |

Table VII collects the results of the absorption spectroscopy experiments, and Table VIII shows the relative values. For each sample, the midpoint temperature was taken to be the point at which the equilibrium constant is equal to 1. The $T_m$ values are similar to those obtained by DSC and UV-CD. The enthalpy for R(ABCDEFG)$_v$ is somewhat higher than that obtained by UV-CD or DSC. The enthalpies for R(AB·CDEFG)$_v$ and R(A·B·CDEFG)$_v$ compare quite closely to the values obtained by the other two techniques. The $T_m$ for R(ABCDEFG)$_v$ is a little lower than that obtained by DSC. The $T_m$ values for R(AB·CDEFG)$_v$ and R(A·B·CDEFG)$_v$ are very close to those obtained by UV-CD. Table IX shows a comparison of the van't Hoff enthalpies obtained from UV-CD and absorption spectroscopy to those obtained from DSC. As in the case of the UV-CD results, the ratio of the van't Hoff enthalpies obtained by absorption spectroscopy to those obtained by DSC seems to indicate little or no intermolecular cooperativity in the denaturation.

## DISCUSSION

The contribution of retinal binding to the stabilty of BR is greater than that of the covalent connection linking helix B to helix C. The contribution is about the same as that of the A–B loop plus the B–C loop. The effect of removing retinal on the $T_m$ and $\Delta H_{cal}$ values was about the same as has been found for vertebrate rhodopsin (Miljanich et al., 1985). The effects of the first and second cleavages on the $T_m$ and enthalpy of denaturation are roughly comparable. If it is assumed that cutting each loop would contribute about equally to lowering the $T_m$, then cutting all of the loops would result in a molecule that would not be expected to be stable at room temperature. Still, individually the covalent links and the retinal binding make rather modest contributions to the stability of the protein, suggesting that side-by-side interactions within the membrane between helices play a significant role in stabilizing the protein.

The effects of cleaving BR once to form R(AB·CDEFG)$_s$ or R(AB·CDEFG)$_v$ and of removing the retinal are to some extent independent. Retinal is able to bind to BR and stabilize it in terms of $T_m$ and enthalpy to about the same extent whether or not the covalent connection in the loop is present. This indicates that the covalent connection is not needed in order for BR to adopt something like its native structure upon binding retinal. Likewise, the connection in the loop stabilizes BR whether or not retinal is present, so retinal is not required for the loop to add to the stability of the protein.

The effect of the elimination of interhelical connections is both enthalpic and entropic, since both the denaturation temperature and the enthalpy are lowered. Thus, the reduction in stability is not simply due to allowing the pieces of the protein to separate from each other more easily because of the lack of covalent links; this would result only in a change in entropy. Rather, there is also an overall loss of stability, indicating that intact loops stabilize the entire structure to some extent, and do not simply hold parts of the molecule together. This fact, combined with the fact that the DSC trace does not contain two peaks corresponding to the separation of the fragments followed by the unfolding of the fragments, indicates that the entire molecule is unfolding cooperatively even when cleaved, again suggesting that interhelical interactions play a role in stabilizing the protein.

Diluting BR with lipid to eliminate the lattice found in purple membrane affects only the $T_m$ values; the relative effects of cleaving and bleaching remain the same. The lattice simply stabilizes the protein entropically. It does not affect the stability of one type of sample more than another, showing that the lattice is not responsible for the association of fragments of the protein.

The intermolecular cooperativity of denaturation remains above 1 when the protein has been cleaved into two or three fragments and when retinal is removed, indicating that the loops and the retinal are not responsible for the association of the molecules. The cooperativity also is not reduced by elimination of the crystal lattice, so the cooperativity is probably due to the trimeric association of the molecules. The denaturation of monomeric BR in micelles shows no intermolecular cooperativity (Brouillette et al., 1989).

The calorimeter traces of the BR samples are asymmetric. The peaks rise gradually on the low-temperature side and drop off rapidly on the high-temperature side. Manly et al. (1985) showed that an asymmetric peak is expected if the native protein is oligomeric and the denatured protein is monomeric. They show that if the unfolding reaction is represented by $N_m \rightleftharpoons mD$, then

$$\frac{\Delta H_{vH}}{RT_m} + (m-1)\ln [N]_0 = \text{constant}$$

where $[N]_0$ is the total initial protein concentration (before heating), so a plot of ln $[N]_0$ versus $1/T_m$ gives a line with

$$\text{slope} = \frac{-\Delta H_{vH}}{R(m-1)}$$

if $\Delta H_{vH}$ is independent of temperature. The present study allows such a calculation because calorimeter measurements

were made on samples both in the form of membrane sheets, with a lipid to protein mass ratio of 1:3, and in vesicles, with a ratio of 2:1. Thus, the protein in the vesicles is at one-sixth the concentration in the sheets. The $\Delta H_{vH}$ values obtained are not consistently higher for either the sheets or the vesicles. If it is assumed, therefore, that the differences in the $\Delta H_{vH}$ values are due only to experimental errors, then $\Delta H_{vH}$ is independent of temperature since the $T_m$ for each sample is consistently lower in vesicles than in sheets. The ratio of ln $[N]_0$ in sheets minus ln $[N]_0$ in vesicles to $1/T_m$ in sheets minus $1/T_m$ in vesicles was calculated, and the values of $\Delta H_{vH}$ in sheets and vesicles were averaged together. From these figures, the value of $m$ (the number of molecules in an oligomer) was found to vary from 2.44 to 3.74, with the value for $R(ABCDEFG)_s$ and $R(ABCDEFG)_v$, the samples which gave the highest quality data, being 2.91. This value of approximately 3 for $m$ is expected for BR, since BR is known to form trimers. Hiraki et al. (1981) found that in $R(ABCDEFG)_s$, BR is still trimeric even above the temperature at which the crystal lattice is lost (around 80°). Thus, the asymmetry of the DSC curves can be explained by BR being trimeric in its native state, while becoming monomeric as it denatures. As further evidence for this, Brouillette et al. (1989) found that calorimeter traces of monomeric BR in micelles are symmetrical. The enthalpy values obtained in this study are therefore consistent with the known trimeric nature of BR.

Manley et al. (1985) give an equation for determining the van't Hoff enthalpy that takes into account peak asymmetry arising from the oligomeric state of a sample:

$$\Delta H_{vH} = ART_{1/2}^2 \frac{C_p(T_{1/2})}{\Delta H(T_{1/2})}$$

where $A$ varies depending upon the value of $m$. For $m$ equal to 3, $A$ would be 7.47. This means that the van't Hoff enthalpies given in Table I should be increased by 87%. The ratio of the van't Hoff to calorimetric enthalpy then becomes approximately 3 for $R(ABCDEFG)_s$ at pH 7 and $R(ABCDEFG)_v$, the two samples for which the best van't Hoff enthalpies can be calculated because of the relative ease of drawing base lines for those samples. The ratio of 3 is the value expected if the cooperative unit of denaturation is a trimer.

Although the denaturation transition of BR is irreversible, it appears that the denaturation of BR may itself be reversible, but after the denaturation occurs, the material undergoes an irreversible transition to a conformation that cannot renature. Sánchez-Ruiz (1992) has reviewed the conditions under which thermodynamic values can be derived from samples that undergo irreversible transitions. If the conversion to an irreversibly denatured state is separate from the unfolding process (that is, occurs after the molecule has unfolded), and if this conversion occurs at a higher temperature than the unfolding temperature and has a small enthalpy, then the fact that the sample denatures irreversibly may not rule out determining thermodynamic values from the calorimeter trace. Freire et al. (1990) calculated a simulation of such a situation, in which the conversion to an irreversibly denatured state occurs rapidly slightly above the reversible unfolding temperature, and found that the scanning rate of the calorimeter does not significantly affect the shape, size, or position of the peak. Thermodynamic values derived from the trace would be essentially the same as if the irreversible step did not occur. Manly et al. (1985) and Edge et al. (1985) obtained similar results for simulated cases. Using this type of reasoning, Sánchez-Ruiz et al. (1988) proposed a general method of determining whether equilibrium thermodynamics can be applied to a transition by determining the midpoint temperature at several scan rates. If the midpoint does not vary with scan rate, then an equlibrium exists in the calorimeter at the midpoint of the unfolding process even though an irreversible step may be occurring at a higher temperature. Brouillette et al. (1987) and Jackson and Sturtevant (1978) found that the shape and position of the peak for BR are not affected by scan rate. This shows that the behavior of BR in the calorimeter is consistent with equlibrium thermodynamics.

As described in the introduction, the work of several other groups shows that each loop is not essential to the structure of BR. In addition, work on other membrane proteins supports the idea that side-by-side interactions between membrane-spanning helices contribute to the stability of membrane protein structure and oligomerization and that extramembranous regions are not essential. Rhodopsin can be proteolytically cleaved in its loops to yield three fragments that remain associated (Litman, 1979). Functional *Escherichia coli* lactose permease can be produced in vivo from genes that yield complementary fragments of the protein (Bibi & Kaback, 1990; Wrubel et al., 1990). The ability of subunits of an IgG Fc receptor to associate is affected by a conservative amino acid substitution within the membrane-spanning region (Kurosaki et al., 1991). The dimerization of glycophorin A depends only on the transmembrane region of the protein (Furthmayr & Marchesi, 1976; Bormann et al., 1989), and its sensitive to the exact amino acid sequence on one face of the helix (Lemmon et al., 1992).

In the introduction, it was proposed that the factors involved in holding the helices of BR together could be assigned to the categories of the loops connecting the helices, polar interactions between helices, the packing of helices with each other versus with lipid, and the binding of retinal. The results given here indicate that individually each connection in a loop or the binding of retinal makes a small contribution to the stability of BR. The stability of the protein is reduced by cleavage or bleaching, but not to the point where the protein unfolds or dissociates into separate fragments. The $T_m$ of each type of BR sample is still well above room temperature.

Although the experiments in this paper do not address the question of whether polar interactions are important in holding the helices together, work by other groups indicates that they probably do not play a major role. A nonpolar residue has been substituted for each polar residue in BR one at a time by changing tyrosines to phenylalanines (Khorana et al., 1988), serines to alanines, and threonines to valines (Marti et al., 1991), and in most cases, the ability of BR to regain its normal spectrum and the ability to pump protons was not affected. When charged residues were replaced with neutral ones, in several cases BR failed to pump protons, but still refolded, showing that these residues are crucial to the mechanism but not the structure of the protein (Stern et al., 1989). Rees et al. (1989) found that very few polar interactions are present within the membrane-spanning portion of the photosynthetic reaction center.

The remaining factor, the packing of the helices with each other, may contribute significantly to the association of helices. Popot and Engelman (1990) have suggested that the free energy penalty of leaving a residue-sized cavity within the membrane may be 1–5 kcal/mol due to unsatisfied van der Waals contacts and the vacuum in the cavity. The free energy stabilization that would result from helices packing with each other could therefore amount to tens of kilocalories per mole of each helix. Yeates et al. (1987) showed that in the

photosynthetic reaction center, the residues in the center of the membrane-spanning portion of the protein are as tightly packed as in the centers of water-soluble proteins. In BR, the packing effect would tend to bring the helices together in a nonspecific way, and the precise arrangement of the helices would depend on the exact arrangement of particular residues, the loops connecting the helices, and the interaction of the protein with the retinal. Side-by-side interactions between helices also appear to play an important role in stabilizing the structure and oligomerization of other membrane proteins.

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