Resonance Raman Evidence for an All-Trans to 13-Cis Isomerization in the Proton-Pumping Cycle of Bacteriorhodopsin†

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ABSTRACT: Using a dual-beam flow technique, we have obtained resonance Raman spectra of the M412 photointermediates of both native purple membrane (15H M412) and purple membrane regenerated with 15-deuterioretinal (15D M412). For comparison, we have also obtained Raman spectra of the *n*-butylamine Schiff bases of the 13-cis and all-trans isomers of 15H and 15D retinal. The 15D model compound spectra, when compared to the 15H spectra, show isotopically induced spectral changes that are markedly different for the two isomers. There is a very close agreement between the frequency and intensity changes which occur upon deuteration of M412 and those which occur upon deuteration of the 13-cis model compound, but not even a qualitative correspondence exists when M412 and the all-trans model compound are similarly compared. These data demonstrate that the chromophore of M412 is an unprotonated Schiff base of 13-*cis*-retinal rather than *all-trans*-retinal. An analogous spectral comparison of 15H and 15D light-adapted bacteriorhodopsin (bR_{LA}) with the 15H and 15D protonated Schiff bases of 13-*cis*- and *all-trans*-retinal demonstrates that bR_{LA} contains an all-trans chromophore, in agreement with previous extraction experiments. Thus, a trans → cis isomerization occurs in the proton-pumping photocycle of *Halobacterium halobium*.

The purple membrane protein, "bacteriorhodopsin", of *Halobacterium halobium* can utilize the energy of visible light absorbed by its retinal chromophore to transport protons against an electrochemical potential across its cell membrane (for a recent review, see Stoeckenius et al., 1979). The bacteria convert the free energy stored by this proton-pumping process to other biologically useful forms of energy by coupling the transport of protons back across the membrane to energy-requiring processes, e.g., the production of ATP[1] (Racker & Stoeckenius, 1974; Danon & Stoeckenius, 1974), and the active transport of amino acids into the cell (MacDonald & Lanyi, 1975).

The mechanism by which light induces proton pumping in purple membrane is only partially understood. Absorption spectroscopy has been used to identify the various kinetic intermediates in the bacteriorhodopsin photocycle (Lozier et al., 1975). The most important features of this cycle are summarized in Figure 1. Besides acting as the initial light absorber, the retinal chromophore probably plays a more direct role in proton pumping; its Schiff base linkage may be an integral part of the pathway along which protons are transported across the membrane. Resonance Raman spectroscopy has shown that the formation of the M412 intermediate from bR_{LA}, which is kinetically associated with release of protons by purple membrane (Oesterhelt & Hess, 1973; Lozier et al., 1975), involves a deprotonation of the retinal–lysine Schiff base (Lewis et al., 1974) and that formation of O from M412, which is kinetically associated with uptake of protons by the membrane, involves a reprotonation of the Schiff base (Terner et al., 1979a).

There is a relatively obvious question about the light-adapted photocycle that has not yet been satisfactorily answered: does retinal undergo isomerization about one of its double bonds during proton pumping, as has been suggested (Pettei et al., 1977; Hurley et al., 1977, 1978; Schulten & Tavan, 1978; Honig et al., 1979)? Such a photoisomerization would be analogous to the 11-cis → all-trans photoisomerization of retinal in rhodopsin. Chromatographic and spectroscopic work on rhodopsin consistently supports the involvement of cis → trans isomerization in the visual mechanism (Wald, 1968; Doukas et al., 1978); however, the corresponding experimental results on bacteriorhodopsin and its proton-pumping mechanism have been ambiguous. While chromophore extraction and reconstitution studies have established that light-adapted purple membrane contains only all-trans chromophores and that dark-adapted purple membrane contains approximately half 13-cis and half all-trans (Dencher et al.,, 1976; Pettei et al., 1977), the isomeric states of the chromophore in the intermediates of the light-adapted photocycle (K, L, M, N, and O) have remained in question.

The M intermediate, which contains the blue-shifted unprotonated retinal Schiff base chromophore, has received the most attention because it is the photocycle intermediate which is easiest to generate in large photo-steady-state concentrations, especially by treatment of purple membrane with ether or with guanidine hydrochloride. Extraction of the chromophore from apparent M intermediates produced under such conditions has yielded anywhere from 60 to 90% 13-*cis*-retinal, the remainder being all-trans (Pettei et al., 1977). Although these data strongly indicate that a 13-cis chromophore is present in M, the experimenters themselves cautioned that it is hard to draw conclusions about native M412 from studies on purple membrane subjected to such drastic treatment. Also, the variability in results could mean that the chromophore in M412 can relax to either 13-*cis*- or *all-trans*-retinal, depending on extraction conditions. A probe of the configuration of retinal that does not require denaturing or otherwise perturbing the protein is needed to solve these problems.

Resonance Raman spectroscopy is well-suited for elucidating the chromophore structures of the intermediates in bacteriorhodopsin's photocycle, because it can selectively give the vibrational frequencies of the chromophore, which are sensitive to changes in retinal's geometric structure. Most importantly, resonance Raman spectroscopy probes the chromophore in situ,

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[1] Abbreviations used: 15D, 15-deuterioretinal; 15H, unsubstituted retinal; OMA, optical multichannel analyzer; RSB, retinal–*n*-butylamine Schiff base; ATP, adenosine 5′-triphosphate; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; high-performance LC, high-performance liquid chromatography.

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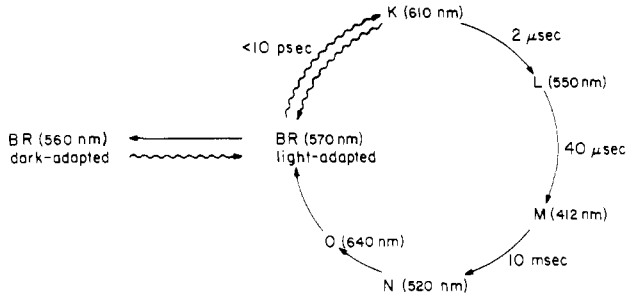

FIGURE 1: Schematic representation of the proton-pumping photocycle of bacteriorhodopsin. Absorption maxima and room temperature decay times are indicated. In the light, bacteriorhodopsin exists predominantly as light-adapted bacteriorhodopsin (bR_{LA}), absorbing maximally at 570 nm. Absorption of a photon by bR_{LA} can convert it to a red-shifted form, K, which decays thermally through a sequence of intermediates (L, M, N, and O) back to bR_{LA}. In the absence of illumination, bR_{LA} converts slowly to dark-adapted bacteriorhodopsin (bR_{DA}).

and it can be used kinetically to study molecules with short lifetimes (e.g., Campion et al., 1977; Marcus & Lewis, 1978). Although resonance Raman spectroscopy has proven to be a very useful tool for determining the state of protonation of the Schiff base chromophore in bacteriorhodopsin (Lewis et al., 1974; Aton et al., 1977), it has unfortunately not been as successful at determining the isomeric state of the chromophore in bacteriorhodopsin photointermediates as it has been for visual pigments. On the basis of essentially identical data, Marcus & Lewis (1978) and Terner et al. (1977) have suggested that the M412 spectrum is closest to that of the *all-trans*-retinal Schiff base (RSB), while Aton et al. (1977) and Stockburger et al. (1979) have opted for 13-cis. This uncertainty results from the relative similarity of the model compound spectra of the all-trans and 13-cis isomers. Isomeric identification by Raman spectroscopy depends most heavily on the number and frequencies of skeletal vibrations observed in the so-called "fingerprint region" from 1100 to 1400 cm^{-1}. For the visual pigments rhodopsin and isorhodopsin, the Raman spectrum of the 11-cis or 9-cis chromophore bound to the protein is sufficiently distinctive to permit unambiguous identification of the isomer (Mathies et al., 1977). For bacteriorhodopsin, the changes in Raman spectrum which result from putting the chromophore into the binding pocket are large enough, compared with the less distinctive differences between the all-trans and 13-cis spectra, that it is impossible to make a clear identification.

In order to establish the configuration of the M412 chromophore, it is necessary to identify those Raman spectral features which result from interactions with the protein binding pocket and those which are characteristic of its isomeric state and are not affected by its environment. We have attempted to assign these features of the M412 Raman spectrum by deuterating the purple membrane chromophore, as well as the 13-cis and all-trans model compounds, at C(15) (see Figure 2) and by studying the behaviors of the vibrations which change as a result of this deuteration. Deuteration at C(15) was chosen because it is relatively simple to carry out synthetically and because the patterns of deuterium shifts in the Raman spectra are distinctive for the two isomers in question. The 15D M412 and model compound spectra demonstrate that the chromophore in M412 is 13-cis.

Materials and Methods

Preparation of Model Compounds. Crystalline *all-trans*- and *13-cis*-retinals (Sigma Chemical Co.) shown to be >97% pure by high-performance liquid chromatography (high-performance LC), were used without further purification. To produce 15-deuterioretinal isomers, we reduced *all-trans*-retinoic acid (Sigma) with $LiAlD_4$ (Alfa) in ether to 15-dideuterioretinol. The retinol was oxidized on finely divided MnO_2 in petroleum ether, and *all-trans*-15-deuterioretinal was obtained from a thin-layer chromatographic separation of the products. A mixture of isomers was prepared by exposing a dilute petroleum ether solution of the 15-deuterioretinal to ambient white light for several hours. The isomers were separated, several milligrams at a time, by preparative high-performance LC on a LiChrosorb Si60 column (Altex) using 10% ether/petroleum ether. The 13-cis and all-trans fractions were shown by subsequent analytical high-performance LC runs to be >97% isomerically pure and by mass spectrometry to be >98% deuterated. Additional samples of 15-deuterioretinal, prepared similarly, were generously provided by Professor J. Lugtenburg, University of Leiden. Retinals were stored as dilute petroleum ether solutions in the dark under argon at −20 °C. Retinal Schiff bases (RSB) were prepared by reacting 10 μL of *n*-butylamine with 1–2 mg of retinal in 1 mL of dry ether at 0 °C for 30 min. The ether and excess butylamine were then evaporated under a stream of argon, and the resulting Schiff base was taken up in CCl_4 for spectroscopy. Protonated retinal Schiff base isomers were separately prepared by acidifying ethanolic solutions of the unprotonated Schiff bases as in Mathies et al. (1977).

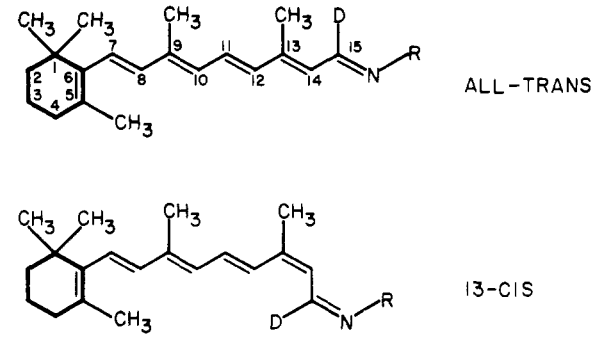

FIGURE 2: Isotopically substituted 15-deuterioretinal (15D) Schiff base isomers.

Preparation of Purple Membrane. Halobacterium halobium was cultured and purple membrane isolated essentially according to published procedure (Oesterhelt & Stoeckenius, 1974). The low-carotenoid strain S9 (obtained from W. Stoeckenius, University of California, San Francisco) was used to minimize the contribution of carotenoid scattering to our Raman spectra. The bacteria were grown in 4-L flasks in a medium containing (per liter of H_2O) 250 g of NaCl, 20 g of $MgSO_4 \cdot 7H_2O$, 2 g of KCl, 0.2 g of $CaCl_2$, 3 g of sodium citrate, trace amounts of $FeCl_2$ and $MnCl_2$, and 10 g of peptone (Inolex).

After harvesting, lysing, and washing the purple membrane until the supernatant in the differential centrifugations was colorless, it was still possible to wash out more of the carotenoid by sonicating the purple membrane. We found that sonication at 80 W (Heat Systems Ultrasonics, W220F) for two 30-s intervals (4 °C), each followed by differential centrifugation, accomplished the removal of carotenoids more completely and consistently than did centrifugation on a sucrose density gradient. Subsequent density gradient purification did not affect the quality or appearance of the resonance Raman spectra. Purple membrane was stored at 4 °C as pellets centrifuged from basal salt solution (the culture medium without peptone).

Raman Spectroscopy. For high-resolution Raman spectra,

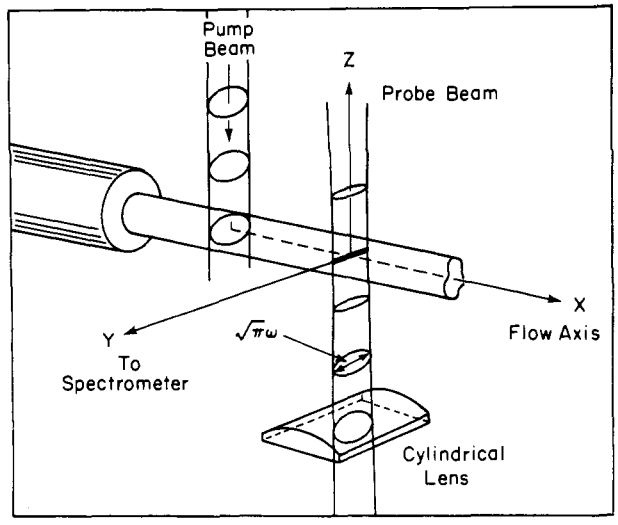

FIGURE 3: Dual-beam flow arrangement used to obtain M412 spectra. The profiles of the two beams are indicated schematically by cross sections: the probe beam is focused cylindrically along the flow axis, while the pump beam is radially symmetric.

we used a Spex 1401 double monochromator with a Spex 1419 illuminator and photon-counting detection (PAR 1105/1120). A microprocessor (Intel 8080) was used to step the monochromator in 1-cm^{-1} increments with a 2-s dwell time. Frequencies are accurate to 2 cm^{-1}.

When more efficient detection was required, we used a modified subtractive dispersion double monochromator system (Spex 1400/1419) with a dry ice cooled intensified vidicon detector (PAR 1205D) and an optical multichannel analyzer (OMA, PAR 1205A) as previously described (Mathies & Yu, 1978). This multichannel Raman system collects data simultaneously in 500 bins extending over ~217 Å (e.g., 700–1750 cm^{-1} for 413-nm excitation). The frequency scales of these spectra were calibrated against Raman lines of benzene and methylcyclohexane to an accuracy of 4 cm^{-1}.

Digital data were averaged and smoothed (three point sliding average) and fluorescence backgrounds (simulated by a quartic polynomial) were subtracted by using a PDP 8/e minicomputer.

Excitation at 413, 514.5, and 676 nm was obtained from a Spectra-Physics 171-01 krypton ion or a Spectra-Physics 165 argon ion laser. Light at 600 nm was produced by pumping a Coherent 590 dye laser with the all-lines output from the argon laser.

M412 Spectra. To obtain spectra of M412, we used a dual-beam flow apparatus (Figure 3) similar to that described by Marcus & Lewis (1978). A small pump (Micropump No. 04-33) recirculated the sample from a reservoir through the illuminated glass capillary (1-mm ID) at ~300 cm/s. Raman spectra were taken with a low-power 413-nm "probe" beam (see Figure 3). An intense 514.5-nm "pump" beam was directed on the entire sample from above, ~0.5 mm upstream from the probe beam, in order to produce a high concentration of M at the probe. Because the probe beam wavelength (413 nm) is very close to the absorption maximum of M412, the scattering of M412 was very selectively enhanced, and almost no scattering from residual bR_{LA} was observed.

The single-pass photoalteration parameter, $F = \sigma\phi P\pi^{-1/2}\omega^{-1}\nu^{-1}$, is a measure of the probability that a chromophore will photoreact during passage through the beam (Mathies et al., 1976). It depends on the absorption cross section, $\sigma = (3.824 \times 10^{-21})\epsilon$, the quantum yield for photoreaction ϕ, the laser power P, the $1/e^2$ radius ω of the laser beam, and the sample flow speed ν. We adjusted P and ω so that the photoalteration parameter of the probe beam was <0.1, even assuming a quantum yield of unity for photoreaction of M, and so that the photoalteration parameter of the pump beam was 1.3, assuming a quantum yield of 0.3 for the photoreaction of bR to M. Under these conditions ~75% of the bacteriorhodopsin was photolyzed to M by the pump beam [see eq 2 in Mathies et al. (1976)]. The small amounts of bR′ (Stockburger et al., 1979) or L′ (Hurley et al., 1978) which could have been formed by the pump beam would not be observed in our spectra because the 413-nm probe beam would not strongly enhance Raman scattering from these species.

The 10-mW probe beam was focused only in the direction the sample was flowing, with a cylindrical lens. This design had the advantage over symmetrical focusing of allowing us to use 100 times as much laser power without increasing the single-pass photoalteration parameter, since the beam radius ω was also increased 100-fold. Focusing along the flow axis does not increase the photoalteration parameter, but the smaller beam waist is imaged more efficiently through the monochromator slit. The short depth of field of the $f/1$ collecting optics meant that photons scattered from regions farther away from the center of the stream were considerably less likely to enter the monochromator. Nevertheless, this arrangement allowed roughly a 10-fold increase in signal levels.

The 0.6-W pump beam was directed onto the sample through a long focal length spherical lens, so that the cross section of the pump beam at the sample was a 1 mm diameter circle whose center was displaced upstream from the probe beam 0.5 mm, corresponding to a time separation of ~0.2 ms. We found empirically that this pump beam configuration gave the greatest M concentration at the location of the probe. Control spectra taken immediately after each M412 spectrum with the pump beam blocked verified that <3% of the Raman scattering in the M412 spectra could be due to residual bR_{LA}. Therefore, no subtraction of bR_{LA} Raman lines was necessary.

The sample reservoir was cooled to 5 °C and illuminated by a 50-W incandescent light to maintain the purple membrane in a light-adapted state. We took spectra by using 25 mL of pH 7 buffered (10 mM Hepes) suspension, at a purple membrane concentration giving an OD of 2 at 570 nm (1-cm path length).

15-Deuterioretinal-Regenerated M412 Spectra. Purple membrane in 1.0 M hydroxylamine was bleached with white light from a 500-W projector lamp and regenerated with a 1.5-fold excess of *all-trans*-15-deuterioretinal (Oesterhelt & Schuhmann, 1974). A modification of the procedure was necessary to avoid the problem of resonantly enhanced Raman scattering from excess retinal added during regeneration and from retinal oxime produced during the bleaching procedure. We extracted most of these contaminants from the regenerated membranes by washing them with distilled water, pelleting them in a centrifuge, lyophilizing the pellet, and extracting it with petroleum ether, before resuspending the purple membrane in water (Tokunaga & Ebrey, 1978). We found that residual amounts of the retinal and retinal oxime could be removed by further irradiation with intense white light, with little destruction of the 570-nm chromophore.

It was also possible to obtain a 15D M412 spectrum without resorting to such treatment of the sample by using a different pump–probe combination: 568-nm light from the krypton laser as pump and 458-nm light from the argon laser as probe. Even at 458 nm, however, the Raman scattering from retinal oxime made a significant contribution to the spectrum, so that it was

necessary to subtract a probe-only spectrum to obtain a pure M412 spectrum.

Except for the bleaching and regeneration procedures, the spectra of 15D M412 were taken under the same conditions as the native M412 spectra. Control M412 spectra, taken of purple membrane regenerated with 15H retinal, showed that these procedures did not affect the M spectrum.

bR_{LA} Spectra. Low-photoalteration native and 15H- or 15D-regenerated bR_{LA} spectra were obtained by using the M412 flow apparatus described above (no pump beam) with a cylindrically focused 15-mW, 514.5-nm probe beam.

M405 Preparation and Spectra. This blue-shifted photointermediate with a several-second decay time was produced under conditions which matched those used in extraction work (Pettei et al., 1977): 2 M guanidine hydrochloride adjusted to pH 9.7 with sodium carbonate solution and a 150-W yellow-filtered spotlight illuminating the sample reservoir. The sample was flowed past a low-photoalteration 413-nm probe beam (without pump beam) to obtain a Raman spectrum with the OMA system. Under the flow conditions used, the sample spent <0.3 s in the flow system between the time it left the completely bleached 5 °C sample reservoir and the time it arrived at the probe beam. This was short enough to ensure that re-formation of bR_{LA} did not occur.

Model Compound Spectra. Raman spectra of the *all-trans*- and 13-*cis*-15H and -15D Schiff bases were taken both at 2-cm^{-1} resolution by using stationary samples in capillaries, red excitation, and the photon-counting system and at lower (6-cm^{-1}) resolution by using 413-nm excitation with the OMA system and flowing sample. For spectra of unflowed samples, ~0.5 mg of model compound isomer was dissolved in 20 μL of carbon tetrachloride, and a 40 mm focal length cylindrical lens focused 30 mW of 600- or 676-nm laser light on the sample. Flowing was necessary in experiments using light at 413 nm, which is strongly absorbed by the retinal Schiff base ($\lambda_{max} \simeq 360$ nm). For these experiments, 0.6 mW of 413-nm light was focused by a 35-mm (spherical) lens onto the sample, which was flowing at 600 cm s^{-1}. Since the resonance enhancements were more favorable at 413 nm than in the red region, it was possible to use lower sample concentrations: ~1 mg of model compound was dissolved in 20 mL of CCl_4. Three OMA spectra, each integrated for 300 s, were taken of each sample. By following the changes in the spectrum with time, it was possible to ascertain that for both stationary and flow spectra, <5% of the scattering was due to photoisomerized molecules. Carbon tetrachloride spectra taken under identical conditions were used to subtract out the solvent Raman lines.

Rapid-flow Raman spectra of the *all-trans*- and 13-*cis*-15H and -15D protonated Schiff bases were taken at 6-cm^{-1} resolution by using 413-nm excitation and OMA detection following the procedures of Mathies et al. (1977).

Results

In Figure 4A,B we present our resonance Raman spectra of 15H and 15D M412. Our 15H spectrum is essentially identical with those previously published (Aton et al., 1977; Marcus & Lewis, 1978; Terner et al., 1979b; Stockburger et al., 1979). However, by using 413-nm excitation, we have eliminated the Raman scattering due to residual bR_{LA} (e.g., a 1530-cm^{-1} shoulder on the ethylenic line and increased intensity at 1200 cm^{-1}) which was observed in all previously published spectra.[2] The 15D M412 spectrum exhibits distinctive isotopically induced changes: the most significant of these are a decrease in the frequency of the 1621-cm^{-1} line to 1604 cm^{-1}, a nearly twofold increase in the intensity of the ~1225-cm^{-1} line relative to the 1180- and 1200-cm^{-1} lines,

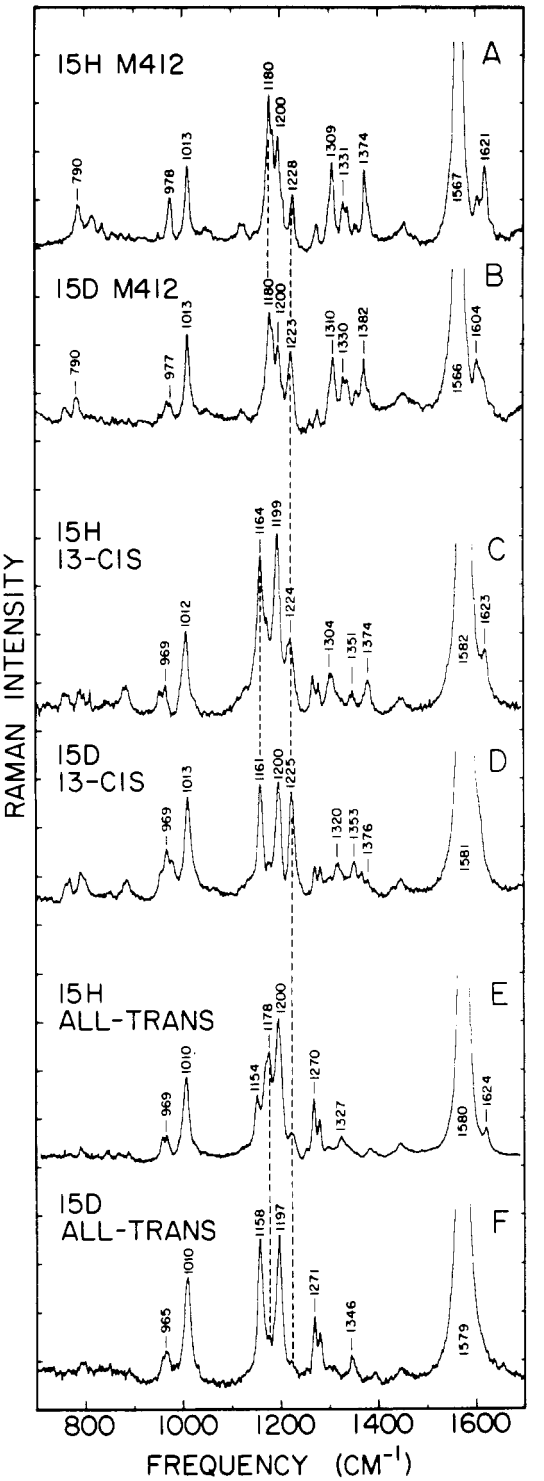

FIGURE 4: Raman spectra of M412 (A and B) and model compounds (C–F) taken with 2-cm^{-1} resolution. (A) Native 15H M412 dual-beam flow spectrum, taken with 600-mW, 514-nm pump and 10-mW, 413-nm probe. (B) M412 regenerated with 15-deuterioretinal, taken under conditions identical with those in (A). (C) Spectrum of a stationary sample of 13-*cis*-retinal Schiff base in CCl_4, with solvent lines subtracted, taken with 30-mW, 600-nm light. (D) 13-*cis*-15-Deuterioretinal Schiff base; conditions identical with those in (C). (E) *all-trans*-Retinal Schiff base; conditions identical with those in (C). (F) *all-trans*-15-Deuterioretinal Schiff base, 10-mW, 676-nm excitation; otherwise conditions identical with those in (C).

[2] If we employ the 458-nm excitation used by previous workers, we also observe these bR_{LA} features. The 15H–15D M412 spectral differences observed by using this excitation are identical with those seen in Figure 4A,B.

and changes in the frequencies of lines around 800 cm^{-1}. The comparisons of spectra of M412 taken before and after regeneration and comparisons of spectra taken of samples that had or had not been subjected to hexane extraction of retinal oximes show that the spectra of Figure 4 do not contain any artifacts resulting from the bleaching and regeneration treatments themselves. Absorption spectra taken before and after bleaching and regeneration showed that over 99% of the chromophores were removed by the bleaching procedure and that ~60% of the bacteriorhodopsin was regenerated when retinal was added to the bleached membranes. Subsequent experiments showed that the incomplete regeneration was due to mild sonication during washing of the apomembrane. This is not significant for our results because our Raman spectra of the M412 and light- and dark-adapted forms of 15H-regenerated purple membrane are identical with those of native purple membrane.

Figure 4C–F compares the spectra of 15H and 15D Schiff bases to the corresponding M412 spectra. The spectra shown were taken with red light (600 and 676 nm) and at 2-cm^{-1} resolution; lower resolution spectra taken at 413 nm (not illustrated) show the same features, although several of the lines close in frequency were not resolved (e.g., the multiple lines in the *all-trans*-15H spectrum at 1154, 1170, and 1178 cm^{-1}). The 13-cis and all-trans model compounds have spectra with clearly different fingerprint regions. The 13-cis spectrum has three strong fingerprint lines at 1164, 1199, and 1224 cm^{-1}, whose frequencies are not significantly altered but whose relative intensities change considerably upon deuteration. The biggest intensity change is that of the 1224-cm^{-1} line, which is markedly larger in the 15D spectrum, compared with the other fingerprint lines. In the all-trans case, however, the opposite trend is seen for the ~1225-cm^{-1} line: the small amount of scattering at this frequency in the 15H spectrum appears to go away in the 15D spectrum. The most notable deuteration-induced change in the *all-trans*-RSB is the disappearance of the peak at 1178 cm^{-1} in conjunction with the increase in intensity at 1158 cm^{-1}. For both 13-cis and all-trans model compounds, the Schiff base vibration at 1623 cm^{-1} disappears upon deuteration. The increase in the reduced mass of the DC=N stretching vibration suggests that this vibration has shifted down under the intense ethylenic line. Features in the spectra near 762 and 791 cm^{-1} may be due to incomplete subtraction of the carbon tetrachloride lines.

Figure 5 compares our spectrum of M405, the guanidine hydrochloride, high-pH photointermediate, to a spectrum of M412. There do not appear to be any significant differences between the two spectra.

Figure 6 compares the spectra of 15H and 15D protonated Schiff base isomers with the corresponding bR_{LA} spectra. Our 15H bR_{LA} spectrum (Figure 6A) is identical with those previously published (Stockburger et al., 1979; Terner et al., 1979b; Marcus & Lewis, 1978; Aton et al., 1977). The fingerprint region of the 15D bR_{LA} spectrum (Figure 6B) exhibits distinctive isotopically induced changes: scattering at 1252 and 1200 cm^{-1} is significantly reduced and a broadening of the 1169-cm^{-1} line is observed upon deuteration. Figure 6C–F compares spectra of 15H and 15D protonated Schiff bases of *all-trans*- and 13-*cis*-retinals. Distinctive deuterium-induced changes are again observed in the fingerprint regions. Deuteration of the protonated *all-trans*-RSB results in a decrease in scattering at 1239 and 1197 cm^{-1} and a slight increase at 1164 cm^{-1} (Figure 6C,D). Deuteration of the protonated 13-*cis*-RSB results in a minor increase of scattering at ~1232 cm^{-1} and a slight decrease in intensity at ~1171 and 1206 cm^{-1}

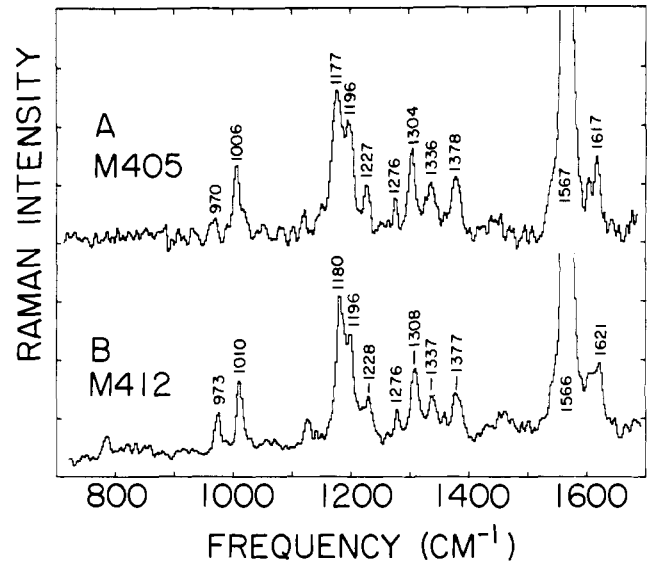

FIGURE 5: Resonance Raman spectra of M405 (A) and M412 (B) taken with 8-cm^{-1} resolution by using 413-nm light and the OMA system described in the text.

(Figures 6E,F).

Discussion

Our 15H–15D comparison method demonstrates that the retinal chromophore in M412 has a 13-cis configuration. Upon deuteration at C(15), the three fingerprint lines in the M412 spectrum at 1180, 1200, and 1228 cm^{-1} exhibit the same spectral changes as those observed for the 1164, 1199, and 1224-cm^{-1} lines in the 13-*cis*-RSB spectrum. Most notably, the ~1225-cm^{-1} lines in both M412 and 13-*cis*-RSB nearly double in relative intensity upon deuteration. Therefore, these modes in M412 and the 13-*cis*-RSB are probably due to similar skeletal vibrations of the retinal chain. On the other hand, the deuteration-induced changes in the M412 spectrum do not agree at all with those observed for the *all-trans*-RSB. The peak in the M412 spectrum at 1180 cm^{-1} cannot correspond to the 1178-cm^{-1} peak in the *all-trans*-RSB, even though both peaks are resolved into two bands at high resolution (Marcus & Lewis, 1979), because in the *all-trans*-RSB, *both* of the lines (1170 and 1178 cm^{-1}) apparently shift upon deuteration to 1158 cm^{-1} (see Figure 4E,F). In M412 the nearly degenerate lines near 1180 cm^{-1} exhibit only small changes upon deuteration. Similarly, the M412 line at 1228 cm^{-1} cannot be due to the same vibration that gives rise to the ~1220-cm^{-1} line in the *all-trans*-RSB because in the model compound the line drops away upon deuteration while in the M412 spectrum the 1228-cm^{-1} line increases significantly in relative intensity. There is thus a large mismatch in the makeup of the fingerprint modes of M412 and the *all-trans*-RSB. The most consistent conclusion is that the chromophore in M412 is 13-cis and that the 1180-, 1200-, and 1228-cm^{-1} lines of M412 correspond with the 1164-, 1199-, and 1224-cm^{-1} lines of the 13-*cis*-RSB.

Our analysis by isotopic substitution assumes that the differences of resonance enhancements and vibrational frequencies between pigment and model compound spectra resulting from binding-pocket interactions are *preserved* when a simple isotopic substitution is made. Further, the frequency *shifts* and intensity *changes* brought about by the isotopic substitution should be similar for pigment and model compound spectra of the same chromophore, even if the spectra as a whole show significant differences. These hypotheses simply say that

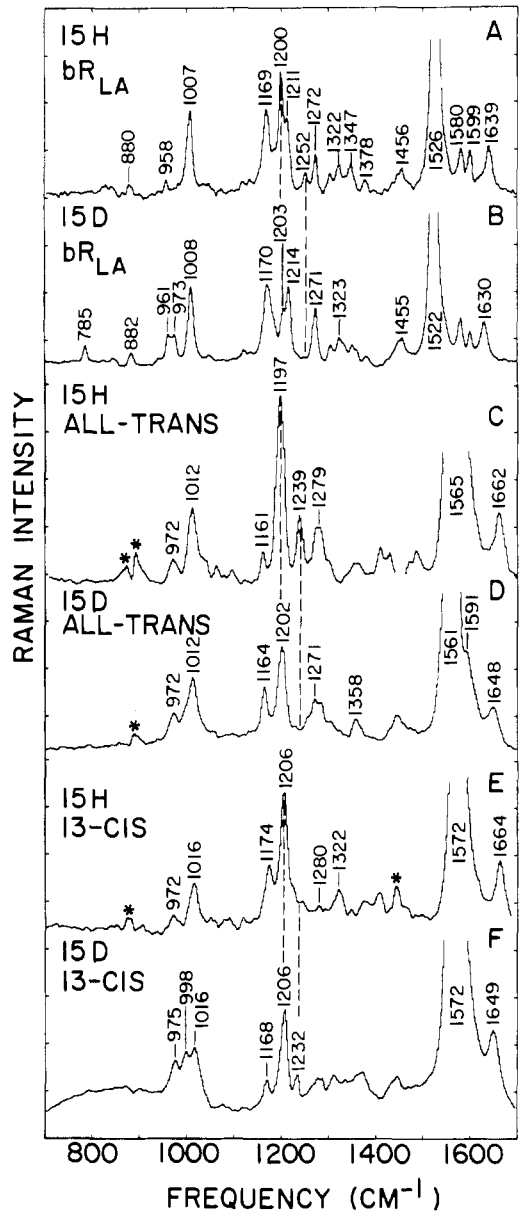

FIGURE 6: Raman spectra of bR_{LA} (A and B) and model compounds (C–F). (A) Native 15H bR_{LA} rapid-flow spectrum taken with 15-mW, 514.5-nm probe and 4-cm^{-1} resolution with photon-counting detection. (B) bR_{LA} regenerated with 15-deuterioretinal, taken under conditions identical with those in (A). (C) Rapid-flow spectrum of all-trans 15H-protonated retinal Schiff base in ethanol (solvent lines subtracted) taken with 0.6-mW, 413-nm probe and 6-cm^{-1} resolution by using OMA detection. All-trans 15D-protonated RSB (D), 13-cis 15H-protonated RSB (E), and 13-cis 15D-protonated RSB (F) taken under conditions identical with those in (C). Solvent subtraction anomalies are denoted by an asterisk.

putting the chromophore in a binding pocket and isotopically substituting it cause perturbations of the Raman vibrational spectrum which are, to a first approximation, additive. The close correspondence between the observed deuteration-induced changes in the 13-*cis*-RSB and M412 spectra indicates that our working hypotheses are correct.

Our data demonstrate that, when the model compound spectra are very similar, the proper matching of lines in the model spectra with lines in the pigment spectra cannot be unambiguously determined by a simple comparison of their number and absolute frequencies alone. Vibrational lines in the model and pigment spectra which have the same frequency may be due to very different vibrational modes, while similar vibrational modes may be observed at significantly different absolute frequencies. It is interesting to note that the frequency difference between the 1180-cm^{-1} mode in M412 and the 1164-cm^{-1} mode in the 13-*cis*-RSB is almost equal to the difference between the 1153-cm^{-1} line in isorhodopsin and the corresponding 1139-cm^{-1} mode in the protonated 9-*cis*-RSB (Mathies et al., 1977). The ~15-cm^{-1} protein-induced shifts caused no difficulty in the configurational assignment of isorhodopsin because the 9-cis fingerprint pattern is much more distinctive than the all-trans and 13-cis fingerprints.

A simple comparison of the *number* of fingerprint lines may also be misleading, since small differences in the spacing and resonance enhancement of vibrations in the pigment and model compound may alter the number of resolved lines. Thus, the splitting of the 1180-cm^{-1} band in M412 could occur because binding-pocket interactions split a nearly degenerate pair of vibrations which together make up the 1164-cm^{-1} line in the 13-*cis*-RSB spectrum. Alternatively, the protein perturbation could increase the enhancement of a line on the high-frequency side of the 1164-cm^{-1} line of 13-*cis*-RSB (e.g., shoulders at ~1175 cm^{-1} in Figure 4C,D).

Our spectrum of M405, a blue-shifted intermediate prepared at high pH and known to have a 13-cis chromophore on the basis of the extraction results (Pettei et al., 1977), confirms our assignment of M412's isomeric state. There are no differences between our M405 and M412 spectra—or even between the portions of those spectra previously published (Marcus & Lewis, 1978)—that are as big as the differences we would expect if these photointermediates contained chromophores that differed by a double-bond isomerization. Our conclusion, from both our M405 spectrum and that previously published, is that they show a chromophore with the same geometry as that of M412.

The 15H–15D spectral comparison for bR_{LA} in Figure 6 is completely consistent with extraction and reconstitution experiments demonstrating that the chromophore in bR_{LA} is all-trans (Pettei et al., 1977; Dencher et al., 1976). Upon deuteration of bR_{LA} and the protonated *all-trans*-RSB, a line in the 1240–1250-cm^{-1} region and a line at ~1200 cm^{-1} lose much of their intensity. Additional scattering is observed in the 1160–1170-cm^{-1} region as a shoulder at ~1180 cm^{-1} in 15D bR_{LA} or as a more intense 1164-cm^{-1} line in the 15D all-trans spectrum. Deuteration of the 13-cis protonated Schiff base (Figure 6E,F) leads to smaller changes with significantly different behavior. Scattering near 1232 cm^{-1} increases while the intensity of the ~1171-cm^{-1} line decreases in the 15D 13-cis spectrum. Thus, the pattern of deuterium-induced shifts for bR_{LA} correlates with the deuterium shifts only in the all-trans model spectra. The most consistent conclusion is that the chromophore of bR_{LA} is all-trans. These Raman data provide an in situ confirmation of the generally accepted idea that bR_{LA} contains an all-trans chromophore. Our isotopic fingerprint method resolves the uncertainty in the determination of the chromophore configuration in bR_{LA} based on Raman data (Stockburger et al., 1979; Terner et al., 1979b; Marcus & Lewis, 1978; Aton et al., 1977). However, because the 15D spectral changes are less distinctive for protonated model compounds than for unprotonated models (see Figure 4), other isotopic modifications with larger changes will be useful in further studies of bR_{LA} and its photoproducts with protonated Schiff base chromophores.

Retinal analogues which regenerate bacteriorhodopsin have been used previously in attempts to assign the spectral features of M412, as well as those of bR_{LA} (Marcus & Lewis, 1978; Marcus et al., 1977). An important difference between the previously studied M412 analogue, containing 3-dehydro-

retinal, and ours is that the former had been modified at the ionone ring end of the molecule, so that the modes which are most distinctive of the 13-cis and all-trans isomers were probably not strongly affected. The modification we used, on the other hand, gives distinctive differences between 13-cis and all-trans isomers, as we have demonstrated by presenting both 13-*cis*- and *all-trans*-RSB spectra. A more fundamental advantage of our deuterated analogue is that it does not cause any change in the chromophore's geometric or electronic structure. Compared to a chemical modification which results in a shifted absorption, a simple isotopic substitution gives a more easily interpretable Raman spectrum, because changes in the spectrum are not due to differences in bond geometries and strengths or different binding-pocket interactions.

Isotopic substitution is a general technique for determining the geometry of any photointermediate whose native Raman spectrum it is possible to obtain. It is necessary only to find the appropriate set of isotopic substitutions which are sensitive to the geometry of the chromophore. These modifications may also give valuable information about the nature of the binding site interactions, but it is not necessary to wait until *all* these interactions are accurately modeled before it is possible to specify the cis–trans configuration of the chromophore. Further isotopic substitution experiments, coupled with normal mode analyses, will be necessary to provide more detailed information about the structure of the retinal chromophore and about chromophore–protein interactions in the proton-pumping photocycle.

The fact that M412 has a 13-cis chromophore has important implications for the light-adapted photocycle. Since bR_{LA} contains an all-trans chromophore, either the bR → K reaction is a *complete* photoisomerization or one of the subsequent steps, K → L or L → M, involves the relaxation of the *photolyzed* chromophore to form an isomerized product. The possible ways in which either type of isomerization could trigger proton pumping have been the subject of much discussion (Lewis, 1978; Warshel, 1979; Honig et al., 1979; Schulten & Tavan, 1978). An attractive hypothesis, consistent with our results, is that isomerization could tranport the Schiff base from an amino acid with a lower p*K*, where the Schiff base would be protonated, to one with a higher p*K*, where it would be deprotonated (Stoeckenius et al., 1979). The Schiff base itself could then serve as one of the proton transporters and as the switch in a "proton wire" extending across the membrane (Nagle & Morowitz, 1978).

Our results, along with the fact that the proton-pumping reactions form a closed cycle (i.e., M412 decays thermally to bR_{LA}), mean that although a *photo*isomerization is responsible for the conversion of bR to M412, the re-formation of bR_{LA} from M must involve a *thermal* isomerization of 13-cis back to all-trans. Such a fast thermal isomerization (complete in milliseconds at room temperature) would require significant catalytic assistance from the protein. One suggested mechanism for such 13-cis to all-trans catalysis in bacteriorhodopsin involves deprotonation of the C(13) methyl group and protonation at the nitrogen, to form an enamine tautomer of the Schiff base with a C(13)–C(14) single bond (Mowery & Stoeckenius, 1979). Evaluation of the geometry of the O intermediate with Raman spectroscopy should help to clarify the mechanism of the reisomerization to all-trans.

Conclusions

The chromophore of M412 is an unprotonated Schiff base of 13-*cis*-retinal while the chromophore of bR_{LA} is a protonated Schiff base of *all-trans*-retinal. Bacteriorhodopsin's chromophore therefore cyclically undergoes isomerization about the 13–14 double bond during proton pumping. Despite a small difference in absorption maximum, the chromophore of M405 has essentially the same structure as that of M412. Comparative Raman spectroscopy of isotopically substituted model compound isomers is a generally useful technique for determining the isomeric state of a retinal chromophore in a protein binding pocket.

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Hemocyanin from the Australian Freshwater Crayfish *Cherax destructor*. Oxygen Binding Studies of Major Components†

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ABSTRACT: Oxygen binding curves have been obtained for unfractionated hemocyanin from *Cherax destructor* and its major components, the 25S and 17S forms. In all cases the binding was characterized by positive cooperativity at pH 7.8 with a P_{50} of ~4 mmHg and a Hill coefficient, n_H, of ~3. There was no evidence of concentration dependence of the binding curves in the range 0.6–6 mg/mL, a finding which excludes a dynamic equilibrium between polymeric forms of different oxygen affinity as a source of the cooperative binding. A positive Bohr effect operates between pH 6.8 and pH 7.8 and removal of calcium ions from the 25S and 17S aggregates markedly reduces their affinities for oxygen. Cooperativity is retained in these circumstances though n_H drops to about 2.5 in the case of the 25S and 2.0 in the case of the 17S form. The two major monomers M_1 and M_2, from which the 25S and 17S complexes are constructed, may be reconstituted into the hexamers $(M_1)_6$ and $(M_2)_6$. These show oxygen binding behavior perfectly consistent with that expected of native hexamers as studied in the 17S fraction, a mixed population of hexamers. The monomer M_1 can also be studied in monomeric form and was found to show indistinguishable oxygen binding at pH 7.8 and pH 10, the curve being a rectangular hyperbola as expected. The oxygen binding curve of the single subunit hexamer $(M_1)_6$ was fitted adequately by a polynomial expression of order 6 as required for a molecule with six binding sites. Further interpretation in terms of a particular binding model was not attempted because available knowledge of the structures of arthropod hemocyanin aggregates and their oxygen binding sites does not yet justify it.

The protein hemocyanin functions in the hemolymph of arthropods to transport oxygen, and the properties of this system are of considerable interest because of the possibility of a complex response arising from the presence of several aggregated forms of the protein, often assembled from a heterogeneous subunit population. For this reason many workers have investigated the oxygen binding of arthropod hemocyanins, some of them using whole hemolymph (Chantler et al., 1973; Loewe & Linzen, 1975; Wajcman et al., 1977) and others using isolated components as well as hemolymph (Sullivan et al., 1974; Terwilliger et al., 1979).

As a result of earlier studies (Murray & Jeffrey, 1974; Jeffrey et al., 1976, 1978), the composition of *Cherax destructor* hemolymph is well understood, at least in terms of its major hemocyanin components, the 17S and 25S aggregates, and the major subunits from which they are constructed. We now report some of the oxygen binding characteristics of *C. destructor* unfractionated hemocyanin and of components isolated from it. The study was undertaken with the aim of relating the oxygen binding behavior of individual aggregated components to their subunit compositions and quaternary structure and ultimately of relating the oxygen binding behavior of the mixture, as it occurs in the animal, to the proportions and properties of the protein species of which it is composed. The present paper documents our progress in this direction to date and discusses our results with *C. destructor* hemocyanin in the context of previous similar studies of other arthropod hemocyanins.

Materials and Methods

Preparation of Hemocyanin Constituents. Pooled serum was prepared from *C. destructor* hemolymph as described previously (Murray & Jeffrey, 1974) and stored under toluene at 5 °C. Individual components were made from the serum by column electrophoresis using the LKB 7900 Uniphor apparatus. The monomer M_1, M_2-rich hexamers, and the 25S components were prepared as described before (Jeffrey et al., 1978). The 17S material was also obtained from Uniphor experiments designed to prepare the 25S component, the first peak from the elution profile consisting of 17S and the second of 25S material. It was found possible to prepare small quantities of the monomer M_2 by column electrophoresis in the same manner as for M_1 and M'_3: the fractions eluting between the M_1 and M'_3 peaks were collected, concentrated, reapplied to the column, and electrophoresed.

Polyacrylamide Gel Electrophoresis and Densitometry of Gels by Scanning. The techniques were carried out as described previously (Jeffrey et al., 1978). Polyacrylamide gel analysis was used to determine the purity and state of aggregation of the solutions used for oxygen binding and to ensure that there was no change in the gel pattern before and after the oxygen binding experiment.

Oxygen Binding Measurements. Oxygen binding experiments were carried out in a tonometer similar to that used by Konings et al. (1969). Since ionic strength has an appreciable effect on the state of aggregation of some hemocyanin components, we have, in general, dialyzed solutions for oxygen binding experiments at an ionic strength of 0.2, with one exception, as described below.

Computing. A nonlinear search routine using the program Fittem, devised by Hay (1968), was used with a Univac 1108

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