Isochromic Forms of Rhodopsin: Isolation and Photochemical Properties†

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ABSTRACT: Rhodopsin was purified chromatographically on ECTEOLA-cellulose, using an aqueous digitonin as solvent. The purification procedure separated three rhodopsin fractions with identical spectral properties. The fraction eluted from the cellulose with buffer (fraction 1) contained about 14 mol of phospholipid per mol of rhodopsin, whereas a fraction eluted at higher ionic strength (fraction 2) contained about 64 mol of phospholipid per mol of rhodopsin. Both rhodopsin fractions migrated with the same electrophoretic mobility on polyacrylamide gel. The regenerability of rhodopsin was 80–90% in fraction 1 and about 30% in fraction 2. The rate constants for the decay of metarhodopsin I at 11 °C were 0.11 s^{-1} for fraction 1 and 5.20 s^{-1} for fraction 2. The low decay rate for the low-phospholipid metarhodopsin from fraction 1 could be due to the prevention by digitonin of conformational changes which take place in opsin during the transition. Thermal intermediates produced by photic bleaching of rhodopsin were studied by low-temperature spectroscopy. The stability of bathorhodopsin was the same whether the intermediate was formed in digitonin or in rods. On the other hand, lumirhodopsin was thermally less labile in digitonin than in rods, although there was no difference in thermal stability whether it was derived from fraction 1 or fraction 2. The results suggest that the first appreciable conformational change of opsin probably occurs in lumirhodopsin.

Photon absorption by rhodopsin in the retinal rod outer segment is the primary event of visual transduction. Rhodopsin is the major integral membrane protein associated with ROS[1] membranes. The pigment has been solubilized with a variety of detergents such as bile salts (Kuehne, 1877), digitonin (Wald and Brown, 1952), Triton X-100 (Crescitelli et al., 1966), CTAB (Heller, 1968), Emulphogene BC-720 (Shichi et al., 1969), Ammonyx LO (Ebrey, 1971), DTAB (Hong and Hubbell, 1972), and alkyl glucosides (Stubbs et al., 1976), and purified successfully by column chromatography in some of the detergents. Rhodopsin solubilized in detergents no longer exhibits the properties of membrane-associated rhodopsin. For instance, upon illumination, rhodopsin in ROS membranes shows a single first-order rate of meta II formation (Applebury et al., 1974). However, each of the thermal intermediates formed from digitonin-extracted rhodopsin decays with multiple rate constants (Abrahamson and Ostroy, 1967). The multiple decay kinetics of intermediates suggests the existence of multiple forms of rhodopsin in this detergent.

To investigate whether the multiple forms of rhodopsin are actually present in digitonin extracts, and also to elucidate what factors are responsible for the decay of intermediates with multiple rates, we have attempted to purify digitonin-extracted rhodopsin by chromatographic methods and have studied the photochemical properties of isolated rhodopsin by flash photolysis. We present evidence that association of different amounts of phospholipid with rhodopsin extracted in this detergent causes formation of the multiple forms of rhodopsin as well as decay of intermediates with multiple rates.

Experimental Section

Materials. Dark-adapted frozen bovine retinas were purchased from George A. Hormel Co., Austin, Minn. Bovine brain PE was obtained from Applied Science Laboratories. The data provided by the company indicated that the brain phospholipid contained the following fatty acids (fatty acid designated by the ratio, the number of carbon atoms:the number of double bonds); 2.3% 16:1, 34.7% 18:1, 15.1% (20:3 + 10:4) and 26.1% (22:5 + 22:6 + 24:1). Bovine PC was also purchased from Applied Science. Ammonyx LO was a gift from ONYX Chemical Co. and CTAB was obtained from Sigma Chemical Co. Digitonin was purchased from Fisher Scientific Company and Sigma Chemical Co.

Isolation of Isochromic Forms of Rhodopsin. Rod outer segments were prepared by the method described previously (Shichi et al., 1969). Aqueous solutions of soluble digitonin were prepared essentially following the method of Bridges (1977) by dissolving 10 g of digitonin in 500 mL of boiling water. The solution was allowed to stand for 2 weeks at room temperature and filtered to remove the solid phase of digitonin. The filtrate was then lyophilized to obtain "soluble digitonin" which, when dissolved in hot water to a given concentration, was more stable. Rhodopsin was extracted from rod outer segments with 2% digitonin in 1 mM potassium phosphate at pH 7.2 and centrifuged at 100 000*g* for 60 min. The rhodopsin-containing supernatant (e.g., A_{498} = 2; 6 mL) was then placed on an ECTEOLA-cellulose column (17 × 1.6 cm) which had previously been buffered with 1 mM potassium phosphate, pH 7.2, containing 0.5% digitonin. About 15 fractions (4 mL/fraction) were collected by elution with digitonin buffer at a flow rate of about 1 mL/min. Elution was continued with a linear gradient of NaCl formed with 100 mL of digitonin buffer and 100 mL of digitonin buffer containing 1 M NaCl. Lyophilized, salt-free rod outer segments were extracted five times with 10 volumes of prechilled *n*-hexane to prepare partially delipidated rod outer segments (Shichi and Somers, 1974). About 30% of the total phospholipid of outer

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[1] The following abbreviations were used: ROS, rod outer segment; CTAB, cetyltrimethylammonium bromide; DTAB, dodecyltrimethylammonium bromide; PE, phosphatidylethanolamine; PC, phosphatidylcholine; PS, phosphatidylserine; meta I, metarhodopsin I; meta II, metarhodopsin II; PI, phosphatidylinositol; TLC, thin-layer chromatography.

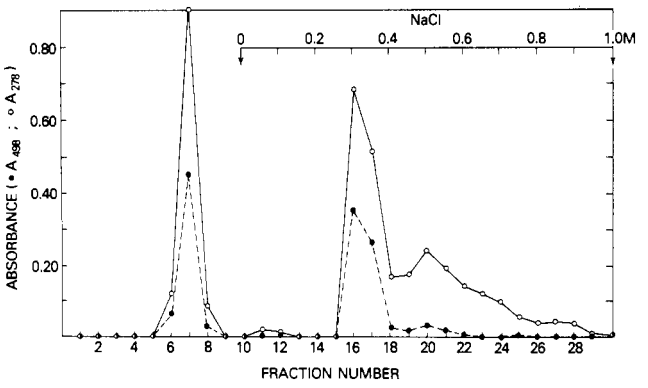

FIGURE 1: Separation of isochromic forms of rhodopsin on ECTEOLA-cellulose.

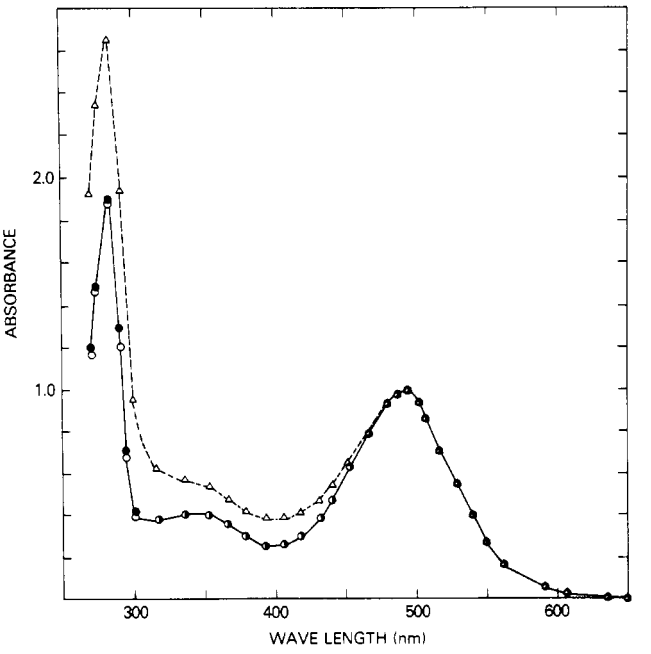

FIGURE 2: Absorption spectra of the isochromic forms of rhodopsin. Spectra were recorded against blank (0.5% digitonin in 1 mM potassium phosphate, pH 7.2) at 20 °C. (○) Fraction 1; (●) fraction 2; (△) fraction 3.

segments were extracted by this treatment. Regeneration of pigment (as isorhodopsin) was carried out by the method described (Shichi and Somers, 1974). The opsin was mixed with threefold 9-*cis*-retinal within 10 min after the bleach (six 40-W cool lamps) and incubated for 90 min in the dark. The optical densities at 278 nm and 498 nm were measured in the dark using a Beckman ACTA II spectrophotometer. Absorption spectra of rhodopsin were recorded with a Cary 14 recording spectrometer at 20 °C and rhodopsin concentrations were determined on a basis of the molar extinction coefficient at 498 nm, μ_M = 42 000 cm^2/mol (Shichi et al., 1969).

Measurement of Thermal Stability of Rhodopsin. Rhodopsin in 0.5% digitonin–1 mM potassium phosphate, pH 7.2, or a suspension of rod outer segments dispersed by homogenization in 1 mM potassium phosphate, pH 7.2, was placed in a cuvette (light path = 1 cm) loaded on a Shimadzu MPS5000 spectrometer, and a change in the optical density at 498 nm due to thermal bleaching was continuously recorded at different temperatures. The temperature at which 50% of rhodopsin had been bleached was determined.

Low Temperature Spectroscopy of Intermediates. For low temperature spectroscopic measurements, rhodopsin in 1 mM phosphate buffer (pH 7.2) containing 0.5% digitonin and 0.1 M hydroxylamine was mixed with 3 volumes of glycerol. The sample was placed in an absorption cell (light path = 5 mm) consisting of a silicon–rubber ring (3 mm in thickness), a front quartz plate, and a back opal glass. The cell was then fixed in the copper sample holder of a specially constructed cryostat (Yoshizawa, 1972) which was subsequently attached to a Hitachi EPS-3T recording spectrophotometer. The light source for flash photolysis of samples was a high pressure mercury lamp (2 K/W, Ushio Co., Japan) and the emission line (>420 nm) was isolated with cut-off and interference filters (Toshiba Co., Japan). The temperature of samples was monitored with a copper–constantan thermocouple. After the absorption spectrum of bathorhodopsin was recorded at −180 °C following a flash bleach of rhodopsin, the sample was gradually warmed to −90 °C to measure the thermal stability of bathorhodopsin. From the elevated temperatures during the warming process the sample was recooled to −180 °C to arrest further decomposition of the intermediate and absorption spectra recorded. The thermal stability of lumirhodopsin was similarly followed at different temperatures. The temperature of the sample that had been warmed was lowered to −80 °C for each spectral recording.

Kinetics of Meta I to Meta II Decay. Rhodopsin in 0.5% digitonin mixed with glycerol and hydroxylamine as described above was placed in a quartz cell (light path = 1 cm) and, after flash photolysis of rhodopsin, decay of metarhodopsin I was continuously monitored at 11 °C by following a decrease in the optical density at 480 nm. The spectral change measured with a Shimadzu MPS5000 was stored in a Hitachi 10-II computer to be retrieved later for analysis.

Phospholipid Analysis. Phospholipids were extracted from dried samples with $CHCl_3$:CH_3OH (2:1, v/v) and separated by TLC according to the standard procedure (Lowenstein, 1969) and the method of Anderson and Maude (1970). After treating the TL plate with I_2 vapor, spots corresponding to the individual components such as PE, PC, PS, lysophospholipids, neutral lipids, etc., were marked and scraped off the plate. The individual phospholipid components were then eluted and digested with 60% perchloric acid. Phosphorus was assayed by the microanalytical method of Hess and Derr (1975).

Polyacrylamide Gel Electrophoresis. Purified rhodopsin was dialyzed at 3 °C for 3 days against 10 mM Tris–acetate buffer (pH 7.4) containing 2.5% sodium dodecyl sulfate and 40 mM dithiothreitol and electrophoresis of protein on 2.5% sodium dodecyl sulfate containing polyacrylamide gel was performed by the method of Fairbanks et al. (1971).

Results

Properties of Purified Rhodopsin Fractions. When digitonin-extracted rhodopsin was chromatographed on an ECTEOLA-cellulose column, three rhodopsin fractions were separated (Figure 1). The first fraction peak (fraction 1) was eluted by digitonin-containing buffer, while the subsequent fraction peaks (fraction 2 and fraction 3) were obtained by elution with a gradient of NaCl at concentrations of about 0.3 M and 0.5 M, respectively. Similar chromatograms were obtained when the pH of phosphate buffer was varied between 6.5 and 7.5, and also when aqueous digitonin (pH 7.2) alone was used. Normalized absorption spectra (OD at 498 nm arbitrarily set at 1) of rhodopsin fractions are shown in Figure 2. Three rhodopsin fractions were spectrally identical and showed the α and β bands at 498 nm and 340 nm, respectively. The A_{278}/A_{498} spectral ratio was 1.9 to 2.0 for both fraction 1 and fraction 2. The spectral ratio for fraction 3 varied in a

TABLE I: Phospholipid Composition of Fraction 1 and Fraction 2 Rhodopsins.

Preparation	Molar ratio of phospholipid to rhodopsin	Mole % phosphorus[a]				
		PE	PC	PS	PI	Unidentified
Fraction 1	14 ± 2	87 ± 8	11 ± 2	ND[c]	ND[c]	2 ± 0.7
Fraction 2	64 ± 5	41 ± 6	42 ± 4	14 ± 2	2 ± 0.5	1 ± 0.1
ROS[b]	ca. 105–126	39 ± 1.2	41 ± 0.8	13 ± 0.8	2.5 ± 0.4	

[a] The values are the mean of 4 determinations ● SD. [b] Anderson and Maude (1970). [c] ND, not detectable.

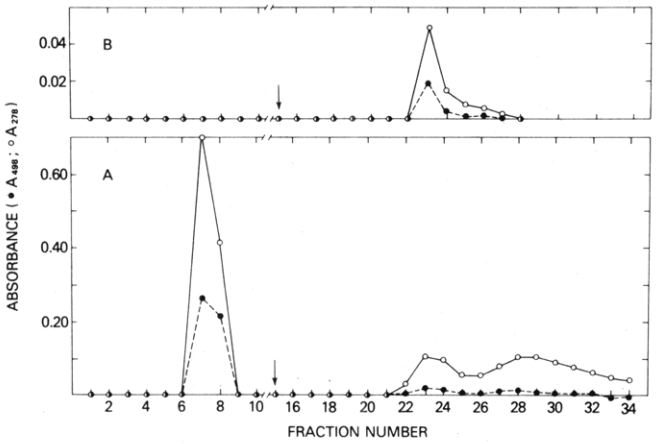

FIGURE 3: Interconversion of fraction 1 and fraction 2 rhodopsin. (A) Fraction 1 prepared by delipidation of fraction 2. (B) Conversion of delipidated fraction 1 back to fraction 2 by reassociation with PE. The arrows indicate the onset of NaCl gradient.

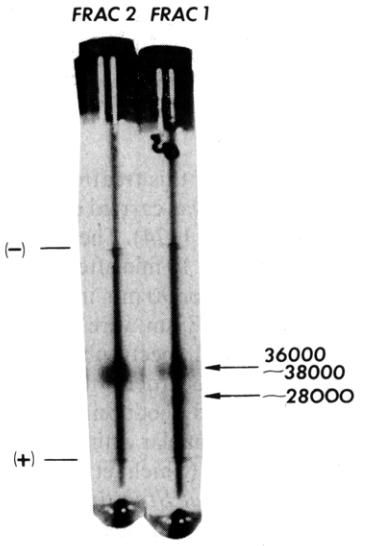

FIGURE 4: Polyacrylamide gel electrophoresis of fraction 1 and fraction 2 rhodopsins. Molecular weight marker (BDH Biochemicals) was used to estimate the molecular weight of proteins separated on the gel. The protein with a molecular weight of ca. 36 000–38 000 corresponds to opsin.

range between 2.6 and 6.0 depending on preparations. Three fraction peaks were obtained also by elution with Emulphogene; two fraction peaks were separated without a gradient of NaCl. With stronger detergents such as Ammonyx LO and CTAB more than 95% of the rhodopsin loaded on the column was collected in fraction 1. Since the sum of fraction 1 and fraction 2 comprises more than 85% of the total rhodopsin recovered in digitonin, and since fraction 3 is a minor component, properties of fraction 1 and fraction 2 only were compared in this investigation.

Chromatographic purification of digitonin extracts from ROS which had been submitted to partial delipidation resulted in a marked decrease in the yield of fraction 2 relative to fraction 1 (data not shown). Since this suggested a possible association of phospholipid with fraction 2, fraction 2 rhodopsin was dialyzed first against 2% Ammonyx to remove phospholipid, then against 0.5% digitonin (pH 7.2) to replace detergent, and chromatographed on ECTEOLA-cellulose. Most of the fraction 2 rhodopsin loaded on the column was collected as fraction 1 rhodopsin (Figure 3A). The fraction 1 rhodopsin thus prepared was then incubated with PE to form rhodopsin phospholipid recombinants (Hong and Hubbell, 1972) and rechromatographed. By reassociation with phospholipid, the rhodopsin collected in fraction 1 was completely converted back to fraction 2 (Figure 3B). Fraction 1 rhodopsin prepared directly from digitonin extracts was similarly converted to fraction 2 rhodopsin by incubation with PE. The "reconstituted fraction 2" contained about 100 mol of phospholipid per mol of rhodopsin. The conversion of fraction 1 to fraction 2 was not specific for PE because PC was effective as well.

To analyze the phospholipid content of the rhodopsin fractions, ROS were thoroughly washed with deionized water, rhodopsin was extracted, and chromatographed with aqueous 0.5% digitonin (pH 7.2) without phosphate buffer. Fraction 2 was found to contain large amounts of phospholipid, whereas the phospholipid content of fraction 1 was relatively low (Table I). The molar ratio of phospholipid to rhodopsin was 64 for fraction 2 and 14 for fraction 1. The extracted phospholipid was cochromatographed with standard radioactive phospholipids and identified by autoradiographic techniques. PE constituted 87% of the phospholipid of fraction 1; 11% of the remaining phospholipid was PC, and PS and PI were not detected. The phospholipid of fraction 2 consisted of 41% PE, 42% PC, 14% PS, 2% PI, and 1% unidentified lipid. Since the phospholipid of rod membranes consists of about 40% each of PE and PC, 13% PS and about 2% PI (Anderson and Maude, 1970), a similar phospholipid composition of fraction 2 suggests that digitonin extraction of this fraction was not selective with respect to phospholipid.

Fraction 1 and fraction 2 rhodopsins migrated with the same electrophoretic mobility on polyacrylamide gel (Figure 4). The molecular weight of opsin was estimated to be 36 000–38 000. Fraction I contained a low-molecular-weight contaminant. Fraction II was essentially homogeneous; though a trace of opsin dimer was detected.

After photic bleaching and subsequent incubation of fraction 1 with 9-*cis*-retinal, 80–90% of the original pigment was regenerated as isorhodopsin. On the other hand, fraction 2 showed no greater than 30% regenerability. The regenerability was not appreciably lost by storage for at least a week at 3 °C. The low regenerability of fraction 2 may be due to peroxidation of associated lipid during the purification procedures. Alter-

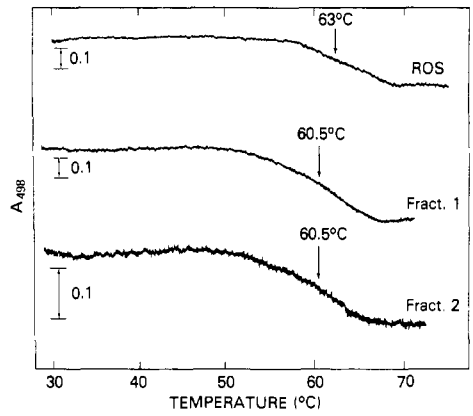

FIGURE 5: The temperature dependence of the optical density at 498 nm of various rhodopsin preparations. The temperature of sample was raised continuously from about 30 °C at a rate of about 2 °C per min.

natively, it may be due to replacement of some of the associated phospholipid by digitonin. In either case the result is a loss of the native conformation of opsin which is required for regenerability (Shichi, 1971).

Thermal Stability of Fraction 1 and Fraction 2 Rhodopsin and Their Bleaching Intermediates. There was no detectable difference in thermal stability between fraction 1 rhodopsin and fraction 2 rhodopsin; the temperature at which 50% of rhodopsin had been thermally bleached was 60.5 °C for both rhodopsin preparations (Figure 5). The comparable temperature for thermal bleaching of ROS-associated rhodopsin was 63 °C. These results indicate that rhodopsin in 0.5% digitonin, regardless of phospholipid content, is less stable to heat than membrane-bound rhodopsin.

The thermal stability of bathorhodopsin and lumirhodopsin generated following flash photolysis of rhodopsin at −190 °C was investigated at low temperatures. Typical results on bovine ROS, fraction 1, and fraction 2 are illustrated in Figure 6. The λ_{max} transiton curves for the decay of bathorhodopsin are essentially identical for fraction 1 and fraction 2 rhodopsin and ROS-associated rhodopsin, with a decay initiation temperature (at which bathorhodopsin begins to decay to lumirhodopsin) at about −150 °C. Therefore, the thermal stability of bathorhodopsin was obviously not affected by detergent extraction of rhodopsin. The thermal stability of lumirhodopsin, whether the intermediate was formed from fraction 1 or fraction 2, was virtually identical and a decay initiation temperature was found at about −30 °C. However, lumirhodopsin generated from membrane-bound rhodopsin was thermally less stable with a decay initiation temperature at −60 °C. Tokunaga et al. (1976) reported similar results on frog lumirhodopsin. The results suggest that the transition of bathorhodopsin to lumirhodopsin involves primarily rearrangement of the chromophore conformation and is least affected by replacement of associated phospholipid by digitonin. Ebrey and Honig (1972) have observed no significant changes of opsin protein in digitonin during the conversion of rhodopsin to lumirhodopsin at −105 °C. On the other hand, the decay of lumirhodopsin is significantly influenced by the molecular environment.

Thermal Kinetics of the Meta I to Meta II Transition. The thermal decay of meta I to meta II followed the first-order kinetics at least for 500 ms after flash photolysis of rhodopsin of fraction 1 as well as fraction 2 (Figure 7). The rate constants measured at 11 °C were 0.11 s^{-1} for fraction 1 meta I and 5.20 s^{-1} for fraction 2 meta I. Meta I from fraction 1 decayed with a first-order rate constant at least for 10 s after the bleaching of rhodopsin. On the other hand, the decay of meta I derived

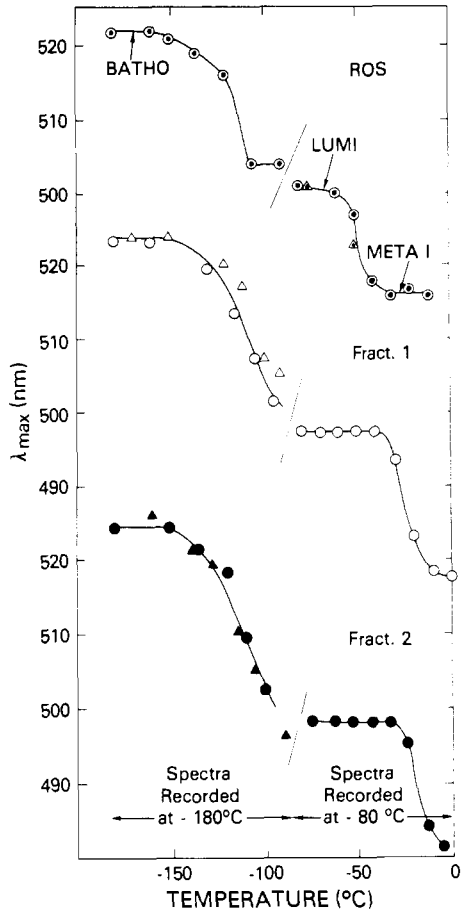

FIGURE 6: The thermal stability of bovine bathorhodopsin and lumirhodopsin as measured by a shift in the λ_{max} at low temperatures. All spectra of bathorhodopsin were recorded after cooling the sample to −180 °C. Spectra of lumirhodopsin were recorded likewise at −80 °C.

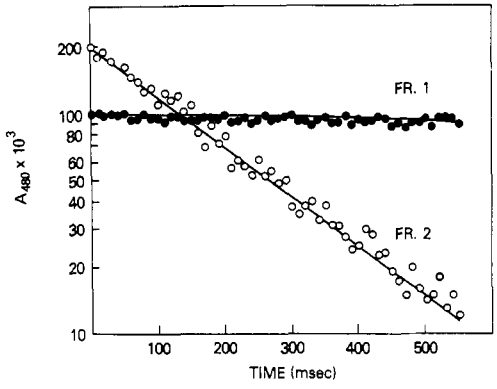

FIGURE 7: First-order kinetic data for the decay of meta I as followed by a decrease in the optical density at 480 nm.

from fraction 2 was of the first order for 500 ms (Figure 7) but gradually deviated from the first-order kinetics as the reaction progressed (data not shown). These results suggest that fraction 1, which is principally a digitonin-rhodopsin complex, decays as a single component, while fraction 2 which contains large amounts of phospholipid, decays as a heterogeneous mixture of rhodopsin species. Because there is an apparent similarity in the phospholipid composition between fraction 2 rhodopsin and membrane-bound rhodopsin (Table I), the kinetic deviation suggests formation of the multiple forms of rhodopsin in this fraction by interactions of digitonin with opsin-associated phospholipid. The transition of meta I to meta

II is reportedly stimulated by the neutral salts lithium bromide and potassium phosphate (Matthews et al., 1963). Since the higher rate constant for the decay of fraction 2 meta I could be due to the presence of NaCl in the fraction, the effect of 0.5 M NaCl (a concentration at which fraction 3 was eluted from the column) on the decay of meta I from fraction 1 was studied. In the presence of salt, the rate constant was somewhat increased (up to 0.18 s^{-1}) but never to the level equal to the decay constant of meta I derived from fraction 2. It is therefore concluded that the higher decay rate of meta I derived from fraction 2 must be due to the association of phospholipid with opsin and not due to the effect of NaCl.

Discussion

A single first-order rate for the meta I to meta II conversion is found for fresh rod outer segments (Applebury et al., 1974) and for retinas in vivo (Hagins, 1957). However, kinetic studies of digitonin-extracted rhodopsin have demonstrated multiple first-order rates for the decay of meta I to meta II (Abrahamson and Ostroy, 1967). Williams et al. (1974) have shown that, while meta I from digitonin-extracted rhodopsin decays with a fast and a slow rate constants, the same intermediate from delipidated rhodopsin decays only with a slow rate. According to these investigators, the addition of PE to delipidated rhodopsin restores the fast rate constant for meta I decay. Therefore, the multiple first-order rates are considered to result from the nonuniform association of phospholipid and detergent with rhodopsin. This view is supported by the present results that the isochromic forms of rhodopsin separated on an ECTEOLA-cellulose column contain different amounts of phospholipid and that the isochromic fractions are interconvertible by association and dissociation of phospholipid. The fact that fraction 2 rhodopsin is eluted in the same chromatographic fractions whether it be associated with PE or PC alone or a mixture of phospholipids argues against a requirement for specific phospholipid. The difference in yields of fraction 2 rhodopsin in various detergents may reflect the difference in the capability of detergent to replace opsin-associated phospholipid. Because fraction 1 and fraction 2 rhodopsins are indistinguishable in the electrophoretic mobility on polyacrylamide gel, the muliplicity of the pigment is not attributed to opsin protein.

The rate of meta I decay seems to be affected both by detergent and phospholipid. The present measurement on fraction 1 and fraction 2 both in digitonin indicates that the lower the content of associated phospholipid, the slower is the rate for the meta I to meta II conversion. O'Brien et al. (1977) have demonstrated that phospholipid unsaturation is essential for the meta I to meta II conversion in rhodopsin–phospholipid recombinants and suggested that a minimum phospholipid bilayer fluidity would be necessary to allow opsin to undergo conformational changes associated with the transition.

When phospholipid content is low, the effect of digitonin will become predominant; the digitonin micelle associated with rhodopsin may form such a rigid environment that opsin conformational change associated with meta I decay is prevented. This will partly explain the slow decay rate of meta I derived from fraction I. Previous observations on different decay rates of metarhodopsin III between fraction 1 and fraction 2 can be explained in a similar manner (Shichi et al., 1977). Since retention of the native conformation of opsin during photic bleaching is essential for regenerability of rhodopsin (Shichi, 1971), the stabilization of opsin conformation by digitonin may also explain the high regenerability of fraction 1 rhodopsin as compared with that of fraction 2 rhodopsin. Circular dichroism measurements indicate that fraction 1 opsin undergoes much less conformational change than fraction 2 opsin during photic bleaching (Shichi, unpublished). The rate of metarhodopsin II formation in CTAB extracts of rods is ten times greater than in digitonin extracts of rod outer segments (Williams and Breil, 1968). The meta I to meta II conversion in dodecyldimethylamine oxide proceeds with a rate 200 times faster than that in sonicated rod outer segments (Applebury et al., 1974). In these strong detergents, opsin conformational change may be accelerated rather than prevented during the meta I to meta II transition.

A comparison of the thermal stability of bleaching intermediates between rod outer segments and digitonin extracts shows that the stability of bathorhodopsin remains unaffected whether it be formed in rods or in digitonin and that lumirhodopsin, on the other hand, is less labile in digitonin than in rods. There is probably a close correlation between spectrally determined thermal stability and opsin conformational stability. These results therefore suggest that the first appreciable conformational change of opsin probably takes place at lumirhodopsin level but not at bathorhodopsin level. The higher thermal stability of lumirhodopsin derived from fraction 1 and fraction 2 than that of lumirhodopsin in rods may also be explained by a suppression by digitonin of opsin conformational changes that would otherwise occur at −60 °C.

It is yet to be determined whether the isochromic forms of rhodopsin isolated in this experiment are identical with the multiple isoelectric forms of rhodopsin reported by Plantner and Kean (1976). In view of the single decay kinetics determined for rhodopsin in rod outer segments (Applebury et al., 1974), the isochromic forms of rhodopsin may well be artifacts produced during detergent extraction. However, it is interesting that three different forms of rhodopsin having different stability toward hydroxylamine have been observed in intact frog retina (Bowmaker and Loew, 1976).

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References

Abrahamson, E. W., and Ostroy, S. E. (1967), *Prog. Biophys. Mol. Biol. 17,* 181.

Anderson, R. E., and Maude, M. B. (1970), *Biochemistry 9,* 3624.

Applebury, M., Zuckerman, D. M., Lamola, A. A., and Jovin, T. M. (1974), *Biochemistry 13,* 3448.

Bowmaker, J. K., and Loew, E. R. (1976), *Vision Res. 16,* 811.

Bridges, C. D. B. (1977), *Vision Res. 17,* 301.

Crescitelli, F., Mommaerts, W. F. H. M., and Shaw, T. I. (1966), *Proc. Natl. Acad. Sci. U.S.A. 56,* 1729.

Ebrey, T. G. (1971), *Proc. Natl. Acad. Sci. U.S.A. 68,* 713.

Ebrey, T. G., and Honig, B. (1972), *Proc. Natl. Acad. Sci. U.S.A. 69,* 1897.

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

Hagins, W. A. (1957), Thesis, University of Cambridge, Cambridge, G.B.

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

Hess, H. H., and Derr, J. (1975), *Anal. Biochem. 63,* 607.

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

Kuehne, W. (1877), *Unter. Physiol. Inst. Heidelberg 1,* 15.

Lowenstein, J. M., Ed. (1969), *Methods Enzymol. 14,* 243.

Matthews, R., Hubbard, R., Brown, P. K., and Wald, G. (1963), *J. Gen. Physiol. 47,* 215.

O'Brien, D. F., Costa, L. F., and Ott, R. A. (1977), *Biochemistry 16,* 1295.
Plantner, J. J., and Kean, E. L. (1976), *Exp. Eye Res. 23,* 281.
Shichi, H. (1971), *J. Biol. Chem. 246,* 6178.
Shichi, H., Lewis, M. S., Irreverre, F., and Stone, A. L. (1969), *J. Biol. Chem. 244,* 529.
Shichi, H., Muellenberg, C. G., Harosi, F. I., and Somers, R. L. (1977), *Vision Res. 17,* 633.
Shichi, H., and Somers, R. L. (1974), *J. Biol. Chem. 249,* 6570.
Stubbs, G., Smith, H. G., and Litman, B. J. (1976), *Biochim. Biophys. Acta 426,* 46.
Tokunaga, F., Kawamura, S., and Yoshizawa, T. (1976), *Vision Res. 16,* 633.
Wald, G., and Brown, P. K. (1952), *J. Gen. Physiol. 35,* 797.
Williams, T. P., Baker, B. N., and McDowell, J. H. (1974), *Exp. Eye Res. 18,* 69.
Williams, T. P., and Briel, S. J. (1968), *Vision Res. 8,* 777.
Yoshizawa, T. (1972), in Handbook of Sensory Physiology, VII/1, Dartnall, H. J. A., Ed., Berlin, Springer, p 146.

Molecular Homogeneity of the Histone Content of HeLa Chromatin Subunits†

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ABSTRACT: Interaction of affinity chromatographically purified antihistone H3 and antihistone H4 with isolated HeLa core particles, followed by separation of unreacted and reacted particles by sedimentation, demonstrates that every core particle contains these histones. Taken together with our previous data indicating the presence of H2B in every nucleosome (Simpson, R. T., and Bustin, M. (1976), *Biochemistry 15,* 4305), these data lead to the conclusion that each core particle contains two each of the four smaller histones. In contrast to the lack of interference in binding of more than one molecule of antibody to a single species of histone to the core particle, steric hindrance exists when attempts are made to bind both anti-H3 and anti-H4 to core particles.

Ample experimental data support the notions that (1) there are nearly equal amounts of DNA and histones in most eukaryotic chromatin; (2) that the four histones H2A, H2B, H3, and H4 are present in nearly equimolar proportions; and (3) that chromatin is organized into a well-defined repeating unit composed of DNA and these four smaller histones. A question which is not fully resolved is whether each nucleosome present in a given tissue is identical in histone content. Recent evidence suggests that some type of heterogeneity in the structure of chromosomal subunits should be expected. Such data include the differential susceptibility of active genes to digestion by DNase I (Weintraub and Groudine, 1976; Garel and Axel, 1976), the presence of posttranscriptionally modified histones (for a review, see Hnilica, 1972), the presence of several species of histone H2A (Laine et al., 1976) and histone H3 (Marzluff et al., 1972; Garrard, 1976) in a single tissue type, changes in the species of histone present during development of a tissue (Cohen et al., 1975; Blankstein and Levy, 1976), and the lack of equimolar ratios of histones in some tissues (Gorovsky and Keevert, 1975).

It seems important, therefore, to devise approaches which will potentially enable us to distinguish between different species present in a mixture of chromatin subunits. Two levels of histone heterogeneity could potentially occur within the core particle: (1) the composition of the protein core could be variable, that is, different ratios of the four core histones might be present in different particles, or (2) all particles might contain two each of the four smaller histones, but there might be sequence variations among these histones in different particles, due, for example, to genetic polymorphism or post-transcriptional modification.

The availability of antibodies specific to purified histone fractions (Stollar and Ward, 1970; Bustin, 1973) which specifically interact with both isolated histones and chromatin-bound histones (Bustin, 1976) allows examination of possible heterogeneity in core particle histone composition at the molecular level. It is possible to examine the histone content of individual nucleosomes by spreading chromatin preparations on electron microscope grids and reacting the spread chromatin with antihistone sera (Bustin et al., 1976). Alternatively, purified nucleosomes can be reacted with purified antihistones and the reaction mixture subjected to sedimentation to separate those particles which have bound the antibody from those which have not (Simpson and Bustin, 1976). Using both these approaches, we have shown that each nucleosome in a tissue contains histone H2B. However, electron micrographs of rat liver nucleosomes reacted with antibodies to the other histones did not confirm the expectation that every particle would react with each specific antihistone antibody. Thus, 27, 54, and 43% of a population of rat liver nucleosomes failed to react with anti-H3, anti-H2A, and anti-H4, respectively (Goldblatt et al., 1977). In view of these results and because of the data of others suggesting possible heterogeneity in nucleosome composition, we have studied in further detail the histone composition of individual chromosomal subunit particles. We have purified core particles from HeLa cells, reacted them with

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