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Evidence for Conformeric States of Rhodopsin

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Abstract. Spectrophotometric measurements of metarhodopsin II appearance are made on five different kinds of rhodopsin preparations. Although the preparations differ greatly in their rhodopsin: phospholipid ratio, the meta II kinetics in all of them are strikingly similar in certain respects. Meta II appearance kinetics in all of the preparations are best described by two and only two exponentials. The ratio of these two rates is always about 5. The fast fraction: slow fraction ratio depends upon temperature. These fractions are reversibly convertible in the dark, and are interconverted on a time-scale which is long compared to the meta II appearance rate. It is shown that the kinetics of the earlier step in the bleaching sequence, viz., lumi- → meta I, is also described by double exponentials. Again the ratio of rates is ca. 5 and the fast-slow fractions correspond to those found in the meta I → meta II step. It is proposed that these facts support an hypothesis for the existence of two conformeric states of rhodopsin which are in thermal equilibrium. Thermodynamic parameters associated with this proposed equilibrium are presented.

Key words: Phospholipid-free – Metarhodopsin II – Double exponential rates – Opsin conformers.

Introduction

The isomerization of 11-*cis* retinal to all-*trans* initiates the bleaching of rhodopsin. The process is remarkable due to the spectral changes which occur: the original, intensely absorbing, purplish pigment is transformed at first into highly colored intermediates, and eventually into the pale yellow substance, metarhodopsin II. The sequence of spectral changes which occur are thought to represent changes in the pigment protein, opsin, which are in turn reflected in the absorption properties of retinal. While there is little disagreement in this field about the *sequence* of the spectral shifts, i.e., bathorhodopsin precedes lumi-, etc., there is not general accord on the mathematical description of the *kinetics* of the spectral shifts (see Discussion).

Recently, results from this lab have indicated that "heterogeneity" exists among the pigment molecules (Williams et al., 1974). It appeared that, upon absorbing light, some of the molecules reached the meta II state faster than others and two exponentials were needed to describe the kinetics. At that time, the apparent heterogeneity was attributed to differences in degree of association of protein and phospholipid because the ratio of fast- and slow-appearing meta II could be manipulated by changing the protein/lipid ratio. However, we have now prepared rhodopsin solutions which are phospholipid-free (Stewart et al., 1976) and have shown in a preliminary report (Stewart et al., 1975) that two forms of meta II still exist in such preparations. Thus, it would appear that the presence of phospholipid is not important for the existence of the forms.

This paper presents data which show that the forms exist in several kinds of detergent solutions and in fresh or frozen rod outer segments. We also show here that lumirhodopsin, an earlier intermediate, exists in these forms.

Materials and Methods

All operations were performed either in darkness or in dim red light. Rod outer segment membranes (ROS) were isolated from frozen cattle retinas (Hormel Company, Austin, Minnesota) by a sucrose flotation procedure (Johnson and Williams, 1970). The pooled ROS were diluted with phosphate buffer and pelleted by centrifugation at 8700 × g several times. If increased purity was desired, the pelleted ROS were again floated on sucrose, collected, and pelleted. The pellet was then either directly frozen, or lyophylized and then frozen.

Sonicated rod outer segments were prepared by suspending the pelleted ROS in 67 mM phosphate buffer, pH 6.5 and adding glycerol to a concentration of 67%. In one case, we obtained fresh, unfrozen retinas and prepared ROS suspensions from these. They were run that same day. The resultant suspension was sonicated in an ice bath using the micro-tip assembly of a Heat System-Ultrasonics, Inc. Sonifier, Model W185. Solutions of the following detergents were all prepared with 67 mM phosphate buffer, pH 6.5. Cetyltrimethylammonium bromide, CTAB, (Sigma) was utilized at a concentration of 1% (w/v). Emulphogene BC 720 was the generous gift of General Aniline and Film Corp., New York, N.Y. and was also used at a concentration of 1% (v/v). A solution of 1.5% (v/v) Ammonyx-LO was made from a 30% stock solution provided by Onyx Chemical Co., Jersey City, New Jersey. All detergent extracts were prepared by homogenization of pelleted ROS, followed by gentle shaking at room temperature for periods of one to four hours depending on the detergent. After centrifugation at 8700 × g for 15 min, the clear supernatant solutions were drawn off and used for spectrophotometric measurements.

Digitonin used in these experiments was prepared as follows: Digitonin (Fisher Scientific) was dissolved in distilled water at a concentration of 10% (w/v) by stirring at 100° C and this suspension was then stored in a refrigerator for several weeks. During this time approximately 50% of the starting material precipitated. The solution was clarified by centrifugation, drawn off, and lyophylized to dryness. The precipitate was discarded. The lyophylized digitonin was ground to a fine powder and stored, tightly capped, at room temperature. Digitonin in this form was found to

dissolve readily, without heating, up to concentrations of 2% (w/v) and such solutions remained clear even during prolonged storage in the cold.

Phospholipid-free rhodopsin was prepared by the method of Plante (cf. Stewart et al., 1976). Dodecyltrimethylammonium bromide (DTAB) was purchased from Eastman Organic and recrystallized three times from 1% (v/v) methanol in acetone prior to use. ROS prepared as above were dissolved in 0.2 M DTAB in 67 mM phosphate buffer, pH 6.8, at a concentration of approximately 30 mg of lyophylized ROS per ml of solution. Phospholipids were separated by gel chromatography at 4° C on a 2.5 × 60 cm column of Bio-Gel A 0.5 (Bio-Rad Laboratories) previously equilibrated with the solubilizing buffer. Digitonin was exchanged for DTAB by adding solid digitonin to the pooled rhodopsin fractions to a concentration of 2% (w/v) followed by dialysis at 4° C against detergent free buffer or distilled water for three days (Hong and Hubbell, 1972).

The phospholipid content of these preparations was determined by extraction of lipid from the detergent solutions using the method of Bligh and Dyer (1959) and determination of lipid phosphorous in these extracts with the modified microprocedure of Bartlett described by Kates (1972). The minimum detectable level of phospholipid using this procedure was estimated to be 0.2 mol phospholipid per mol rhodopsin. The rhodopsin content of the solutions was determined spectrophotometrically using a molar extinction coefficient for rhodopsin at 500 nm of 40,000 l/mol-cm.

A rapid recording flash-photolysis spectrophotometer (frequency response D.C. to 1 MHz) was designed and constructed for use in these experiments. This apparatus incorporates some features of an instrument previously described by Williams (1968) and other features of an instrument described in detail by Marquisee (1966).

A 0.2 ml sample of solution in a 1 cm-path semimicrocuvette was flashed from the side while the transmittance of the solution was continuously monitored in the perpendicular direction. The flash bleached no more than about 23% of the rhodopsin in a sample. The cuvette containing the sample was enclosed in a specially constructed sample compartment whose temperature was controlled to within ± 0.2° C by circulating water through the hollow body of the compartment with a thermostated circulator. Sample temperature was measured by temporarily immersing a thermistor in the sample prior to photolysis.

The experimental procedure was as follows: a cuvette containing the sample is placed in the sample compartment, allowed to come to thermal equilibrium and its temperature checked. At this point, in rapid succession (1) a photometric calibrating filter was inserted into the monitor beam and the oscilloscope triggered and photographed, (2) the calibrating filter was removed and the oscilloscope triggered and photographed again, (3) the flash lamp was fired and the oscilloscope automatically triggered synchronously and photographed, and (4) after approximately 5–30 sec, depending on the sweep speeds used, the oscilloscope beam was again triggered and photographed. The change in transmittance relative to the preflash value can be calculated from the deflection caused by insertion of the calibrating filter. Displacements proportional to transmittance were obtained from the oscillograms by direct measurement to the nearest 0.1 mm. Dual oscilloscope traces were used with independently varied time bases so that fast and slow rates were displayed with optimal resolution.

All calculations were done on a Hewlett-Packard 9810A Programmable calculator. The 9810A was programmed to calculate absorbance, A and $(A_\infty - A)/A_\infty$ as a function of time, given the displacement as a function of time and the scale factor relating transmittance to displacement. First order relaxation times were calculated by least squares fit of straight lines to log $[(A_\infty - A)/A_\infty]$ as a function of time using the "peeling-off" procedure. That is, this function is assumed to be of the form $\Sigma_i A_i \exp(-t/\tau_i)$. A straight line was fitted to selected points in the "tail" portion of this function and the time constant and amplitude of the slowest relaxation process calculated from the slope and y-intercept of this line. The y-coordinates of points on the line were subtracted from y-coordinates of the corresponding data points yielding a second function to which a second straight line was fit, and so forth.

Results

This section is subdivided into two parts. The first deals with kinetic evidence for the existence of two forms of the intermediates. The second part is based on the hypothesis that rhodopsin, itself, exists in the two forms, and that these forms are in

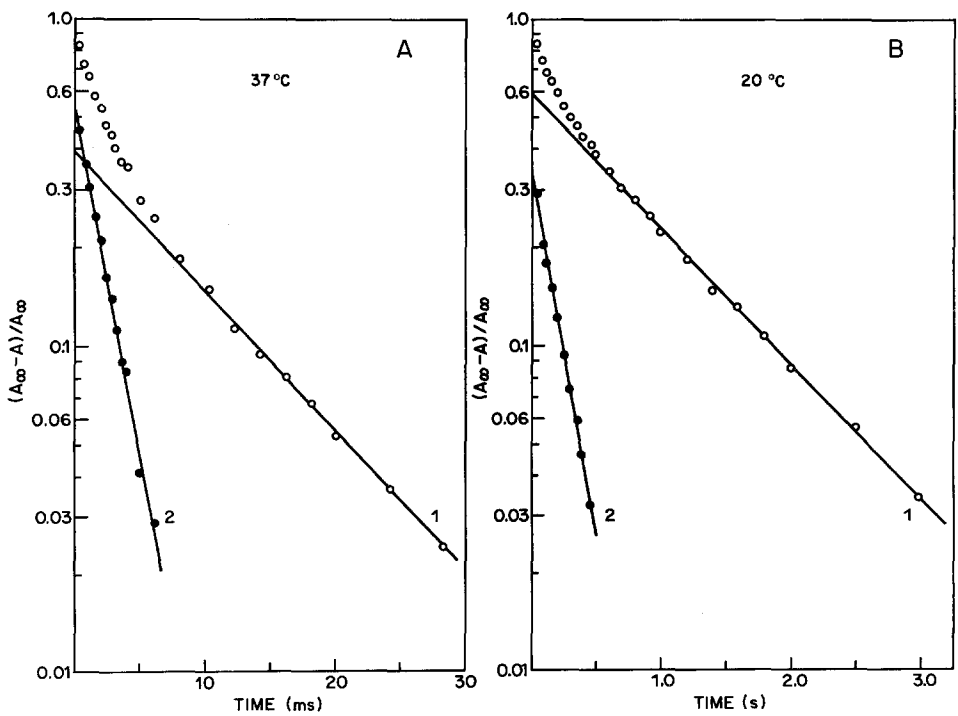

Fig. 1. Part A shows the double exponential kinetics of meta II appearance at 37° C in phospholipid-free preparation. Open circles are the original data from which the slow rate constant, k_1, is obtained directly. Filled circles are derived as described in Methods and permit the calculation of the faster rate constant, k_2. The ratio k_2/k_1 is 5.0. The intercepts give A_1 and A_2, the fraction of meta II appearing with the slow and fast rates, respectively. About 60% is fast and 40% slow at this temperature. Part B. Meta II appearance in phospholipid-free preparation at 20° C. Two rates are extracted and the ratio of fast- to slow-appearing meta II is 40/60 at this temperature. The ratio of rates is 5.8

thermodynamic equilibrium. In the latter part are given the thermodynamic properties of this proposed equilibrium.

Figure 1 shows an integrated, first-order rate plot of the appearance of meta II at two temperatures. This preparation is phospholipid-free rhodopsin in 2% digitonin, 0.67 M phosphate buffer, pH 6.5. The overall rate (open circles) is comprised of two, separable first-order rates. The slopes of the lines give the rate constants for the fast and slow rates and the intercepts, the fraction of meta II appearing at these rates. We define the intercepts, A_1 and A_2, for the slow and fast rates, respectively, and we find that the sum, $A_1 + A_2$, is always equal to 1.0 within experimental error. This shows that only two first-order rates are needed to define the entire process. The A_1/A_1 ratio depends upon temperature: in Figure 1 it is about 60/40 at the higher temperature and 40/60 at the lower. This effect of temperature on the A_2/A_1 ratio is reversible: If, in the dark, a sample is brought to 21° C, then raised to 37° C and again brought to 21° C and finally flashed, the results are identical to those obtained from a sample at 21° C which had not been taken to 37° C. Since it takes several minutes for complete reversibility at low (say, 0°–10° C) temperatures, it should be possible to study the kinetics of this process, but we have not yet done so.

Figures 2 and 3 show meta II production in ROS and in CTAB. Opsin in ROS is associated, of course, with much phospholipid as it is, perhaps, to some extent in CTAB. Nevertheless, two, and only two, rates are needed to describe the meta II

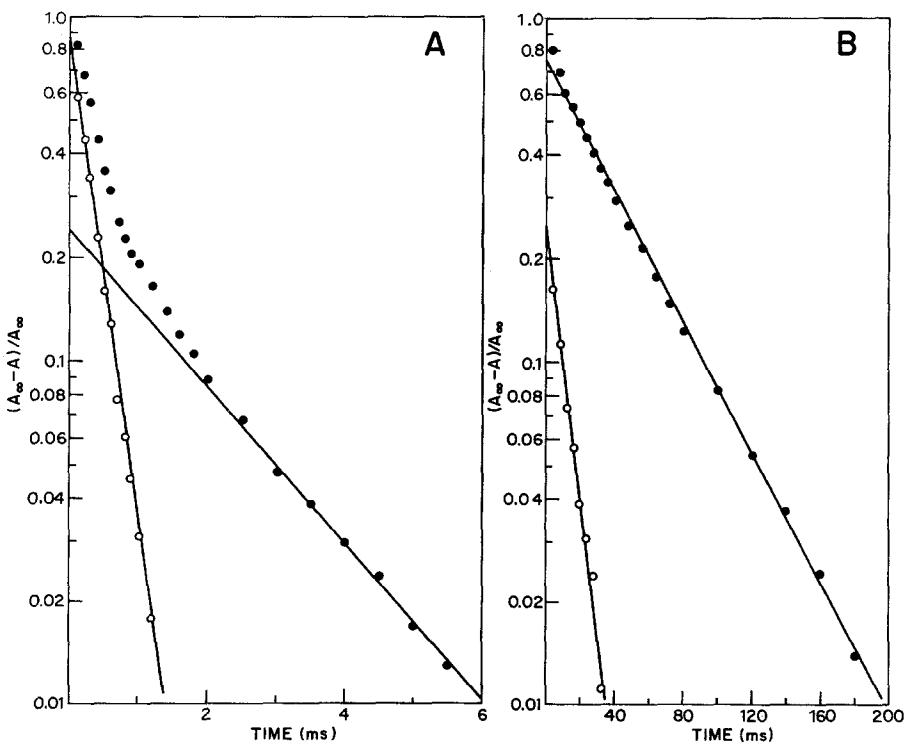

Fig. 2. Meta II appearance in sonicated ROS. On the left, two rates obtained at 37° C and are in the ratio, 6.0. A_1 and A_2 are ca 0.20 and 0.80, respectively. On the right, the ratio of rates is 4.7 at 20° C while A_1 and A_2 are 0.75 and 0.25, respectively

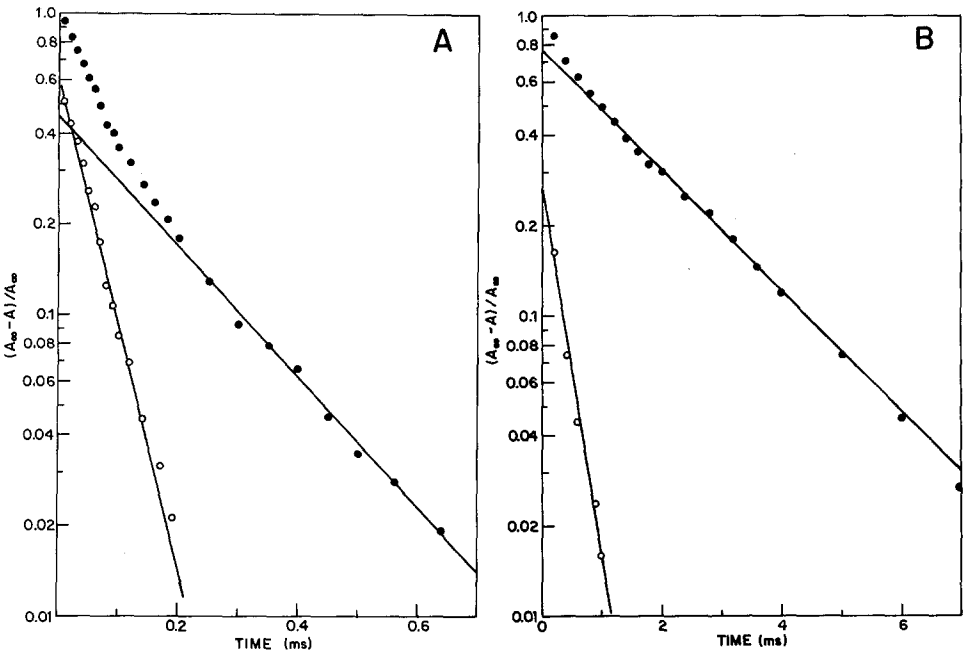

Fig. 3. Meta II appearance in CTAB preparations. On the left, the ratio of rates at 25° C is 4.0. A_1 and A_2 are 0.45 and 0.55. On the right, the ratio of k's is 6.2 at 10° C. A_1 and A_2 are 0.73 and 0.27

Table 1. Activation Parameters for meta II production

Preparation		$\Delta H^{\neq}$ kcal/mole	$\Delta S^{\neq}$ eu	$\Delta G^{\neq}$ kcal/mole (20° C)
Sonicated ROS in	fast k	37.7	65.5	18.4
67% glycerol buffer, pH 6.5	slow k	37.1	60.5	19.4
Phospholipid-free rhodospin	fast k	53.0	125	16.0
in 2% digitonin, pH 6.5	slow k	54.0	125	17.0
ROS solubilized in	fast k	29.0	45.9	15.5
1% CTAB, pH 6.5	slow k	31.1	50.1	16.5

appearance. Other kinetic data, obtained from preparations of ROS dissolved in Emulphogene BC 720 but not presented here, also show the two rates.

Thus, despite the fact that we have used preparations which vary greatly in their lipid/opsin ratio, we always find two rates of meta II appearance. Furthermore, the ratio of rate constants, k(fast)/k(slow) is always about 5/1. And, over the temperature range, 0°–40° C, the A_2/A_1 ratio is never greater than 10/1 or less than 1/10.

Table 1 summarizes the rate parameters found in the three preparations.

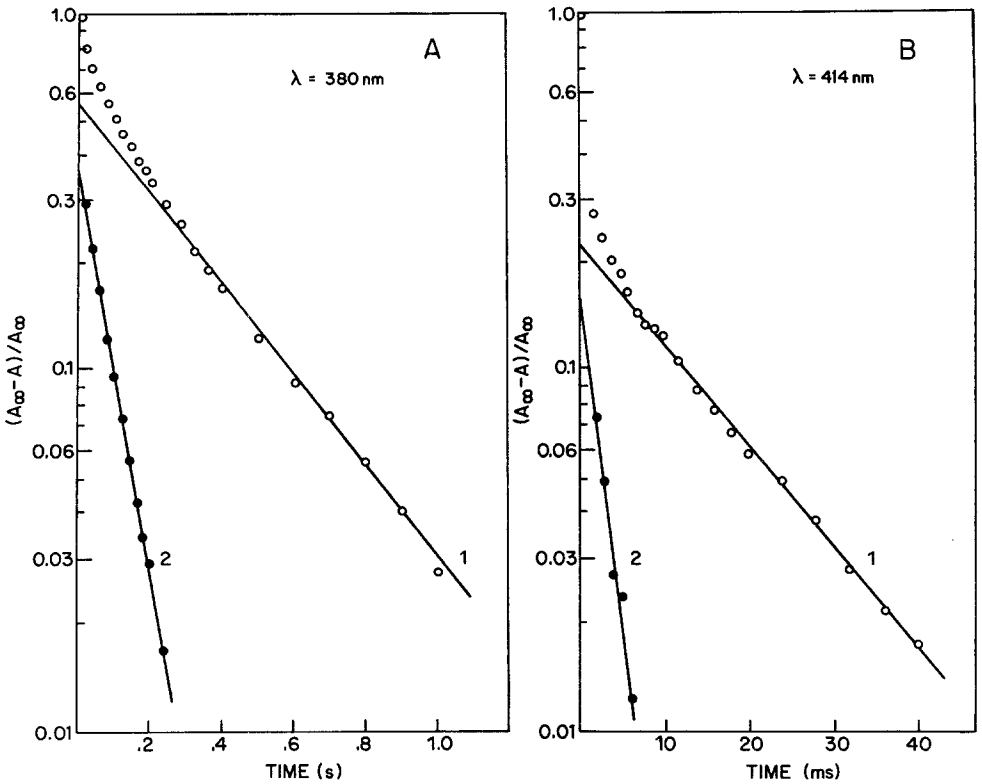

Fig. 4. Comparison of meta II appearance kinetics with those of lumi-decay. Part A. Meta II appearance at 22° C in phospholipid-free preparation. Two rates in the ratio, ca 5, are resolvable from the original data and the ratio of fast to slow appearing meta II is ca 60/40. Part B. Kinetics of lumi-decay (meta I appearance) measured at 414 nm. After an "instantaneous" increase in absorbance due to the very rapid production of lumi-, the decay of lumi-follows double exponential kinetics. The ratio of rates is again ca 5 and the ratio of fast- to slow-decaying lumis is also ca 60/40

An important question which remains is: how early in the bleaching sequence can the two rates be observed? So far, we have only reported on the meta I → meta II reaction. Now we show in Figure 4 that lumi- → meta I proceeds with two parallel rates. The *A*-part of this figure shows the production of meta II for comparison. Meta I appearance is measured at the isosbestic of the meta I-meta II reaction, 414 nm. At this wavelength, lumi-absorbs more strongly than does rhodopsin, so that, upon flashing, there is an "instantaneous" increase in absorbance as lumi- appears. Thereafter, the absorbance increase is a composite of lumi-decay and meta I appearance. (The observed kinetics are not altered by this spectral overlap.) The overall rate is again comprised of the two first-order processes. About 60% of the meta I appears slowly and 40% quickly. These same proportions describe the fast and slow components of meta II appearance in part *A* of this figure. Since we have some indication that the time for interconverting the fast and slow forms is long compared with the appearance of meta II, it would seem that the fast-decaying lumi-

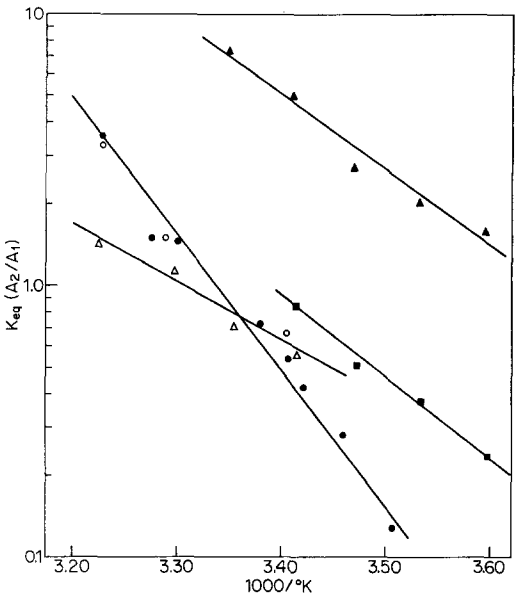

Fig. 5. Van't Hoff plot of proposed equilibrium between rhodopsin conformers. Filled triangles: Emulphogene preparation. Squares: CTAB preparation. Open circles: ROS from fresh (never frozen) retinas. Filled circles: ROS from previously frozen, stored retinas. Open triangles: phospholipid-free rhodopsin in 2% digitonin, distilled H_2O, pH 6.0

Table 2. Thermodynamic parameters for equilibrium of rhodopsin conformational states

Preparation	$\Delta\bar{H}^0$ kcal	$\Delta\bar{S}^0$ eu	$\Delta\bar{G}^0$ kcal (37° C)
Phospholipid free rhodopsin in 2% digitonin, distilled H_2O, pH 6.0	9.8	32	−0.2
ROS solubilized in 1% CTAB, pH 6.5	13.5	45.9	−0.7
ROS solubilized in 1% Emulphogene BC-720, pH 6.5	12.6	46.2	−1.0
Sonicated ROS in 67% glycerol-buffer, pH 6.5	21.9	73.2	−0.8

molecules are the same ones which decay through meta I and show up as fast-appearing meta II.

With our present apparatus, we are unable to study the batho- → lumi-reaction; it is too fast. However, Grellmann et al. (1964) reported that in ROS they found two rates in the batho- → lumi-step. Taken together with our measurements, this indicates that the heterogeneity exists in the parent pigment, rhodopsin, itself. This leads us to hypothesize that two forms of rhodopsin exist and that they are in equilibrium with each other. When these forms absorb light from a flash they bleach at rates which differ by a factor of about 5 but both of these rates, down to and including the meta I-meta II step, appear to be faster than the time it takes to interconvert the forms. If this is true, then measuring the fast- and slow-appearing meta II is tanta-

mount to measuring the amount of rhodopsin which existed in the two forms before the flash. The ratio of the meta II's, A_2/A_1, is thus the equilibrium constant for the proposed rhodopsin interconversion.

Figure 5 is a van't Hoff plot of the results obtained from the five preparations we have used. (Note: open circles were obtained from unfrozen cattle retinas less than one day old.) They all give ΔH values in the range 10—20 kcal/mole. Complete sets of thermodynamic parameters are given in Table 2 and do not differ very much across widely different preparations. This observation suggests that the changes in the state functions of the proposed forms do not depend strongly on the environment but rather that they reflect some internal change in opsin. Perhaps rhodopsin exists in two conformeric states.

Discussion

We have questioned the validity of treating the rate data as the sum of two exponentials and have tried other single, higher-order, treatments. None of these worked as well as what we have presented here. In addition, no dependence on the rhodopsin concentration of the rate constants was ever observed. Thus, a higher order dependence on rhodopsin, say second-order, is experimentally ruled out. With one exception (Rapp et al., 1970) other investigators have also concluded that the kinetics should be treated as a first-order or a set of parallel, first-order reactions (Linschitz et al., 1957; Wulff et al., 1958; Grellmann et al., 1962; Abrahamson et al., 1960).

There are reports in the literature which indicate that more than two separable first-order rates are needed to define the bleaching steps (Abrahamson et al., 1960; Ostroy et al., 1966; Pratt et al., 1964; and Wulff et al., 1958). It turns out that in these cases in which 3 or 4 rates are reported, it is always true that the preparation is phospholipid-containing ROS solubilized in digitonin. We, also, have observed as many as 4 rates in this particular preparation but in no others. It appears that the 4 rates can be categorized as two sets of two rates each, but we have not actively pursued a more complete description of these observations. Instead, we have chosen to work on the simpler and, what appears to be, more general case of two rates.

Still others (Applebury et al., 1974; Rapp et al., 1970; Sengbusch and Stieve, 1971) have reported that only one first-order equation adequately defines the rates of some of the reactions. Here, again, the explanation may lie in the nature of the preparation that was used. The detergent in which a single exponential has been found is LDAO (Applebury et al., 1970). We have run a few experiments in ALO, a system similar to LDAO, and see only one rate at temperatures above 10° C but *two* at lower temperatures. Perhaps these particular detergents shift the proposed equilibrium toward the fast conformer even more than does Emulphogene BC-720 (see Fig. 5), and only at low enough temperatures can any appreciable amount of slow form be seen.

The hypothesis that two conformeric forms of rhodopsin exist can be tested. Microcalorimetry might disclose their presence: one would determine the enthalpy change over the range of temperature reported here and compare it with our ΔH

values. Alternatively, changes in CD or ORD might be sought. In general, some test which is independent of the kinetics of bleaching should be applied in order to verify the existence of the forms. For purposes of the present discussion, we shall assume that they do exist.

How extensive would be the proposed change in opsin in order to account for the observed ΔH and ΔS? The theory of conformational changes in proteins is currently the object of serious study but unfortunately no unambiguous answer to this question exists. One moderately successful approach to this problem is a modification of the one originally proposed by Kauzmann (1959) for protein denaturation. The idea here is that denaturation (or conformational change) exposes previously "covered" hydrophobic amino acid side chains to a polar environment. Associated with the transition of each side chain into the new environment are particular ΔH's and ΔS's whose numerical values can be estimated from separate experiments on the individual amino acids. When we use average values of these parameters we find that the *lower limit* implied by our results is that no more than about 7 hydrophobic residues need be exposed. However, we can say nothing about the real case since, for example, we do not know which particular residues are exposed or if compensatory changes simultaneously occur or if other than simple hydrophobic effects are involved.

The values of ΔH, ΔS and ΔG we observed for the hypothetical transition are not very different from others that have been measured for various conformeric transitions in α-chymotrypsin and ribonuclease, two well-studied proteins (cf. Kim and Lumry, 1971; French and Hammes, 1964). The roles played by the conformers in these proteins are not understood and even if they were, conclusions drawn by analogy with them may be misleading since neither protein is part of a membrane system. Hence, little can be said about possible functions served by two conformers of rhodopsin. However, it is clear that two rates exist in fresh ROS, our most "physiological" preparation, and that both of the forms exist in appreciable quantities at physiological temperatures. Hence, it may be that they do play roles and perhaps these roles will become discernible in the near future say in studies of light- and dark-adaptation.

There is another possible interpretation of the results we have presented: the apparent heterogeneity of the rhodopsin could be a result of the heterogeneous *environment* of the rhodopsin which in turn would be to due to the presence of mixed lipid-detergent micelles. This explanation requires that there be two classes of micelles which represent two distinct phases in the aqueous environment. The extent to which each type is present would depend upon the concentrations of the lipids and detergents as well as temperature. Thus, this alternative would emphasize the importance of the lipids and detergents but would ignore the possibility that conformers of opsin exist.

However, we hasten to point out, in countering the above alternative, that some of the preparations had no detergent (ROS and fresh ROS) while others had no phospholipid (cf. Fig. 1). Furthermore, preparations in CTAB, Triton, Emulphogene and ALO all leave varying amounts of phospholipids associated with the opsin and even so two *and only two* forms are found. Hence, for these reasons, it is difficult to accept the alternative explanation. Furthermore, our digitonin no doubt is impure (a mixture of saponins) but when all phospholipids are removed from the opsin *only*

two rates are seen. When no lipids are removed and ROS are simply dissolved in digitonin, up to *four* rates are seen. This case would seem to conform to the notions of the mixed micelle arguments but it is the *exception* in our experience. As previously stated we have chosen not to investigate it because of its apparent complexity and because it seems to represent a special case.

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