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TWO DISTINCT RHODOPSIN MOLECULES WITHIN THE DISC MEMBRANE OF VERTEBRATE ROD OUTER SEGMENTS

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Summary

The kinetics of the metarhodopsin I—II reaction have been measured over a wide range of temperatures (1—37°C) and pH values (4.5—8) with suspensions containing fragments of bovine rod outer segments. It was found that for all conditions the occurrence of metarhodopsin II could be described by two independent first-order processes. The fast component : slow component amplitude ratio depends upon pH and temperature.

The kinetics of the lumi-metarhodopsin I reaction show the same pH dependence for the fast component : slow component amplitude ratio as the one observed for the metarhodopsin II signals.

All the results observed could be described with the assumption that rhodopsin itself exists in two conformational states before bleaching which are in a pH and temperature-dependent equilibrium. This hypothesis is confirmed by its ability to explain some apparently anomalous observations in the literature.

Introduction

The molecular photoreceptor in both vertebrate and invertebrate rod cells of the retina has been shown to be the chromoprotein rhodopsin. It consists of a membrane-bound protein, opsin, and a covalently bound prosthetic group, 11-*cis*-retinal. Following light absorption a series of intermediate steps have been spectrally defined for this chromoprotein. The intermediates which have characteristic absorption maxima (specified at room temperature) are summa-

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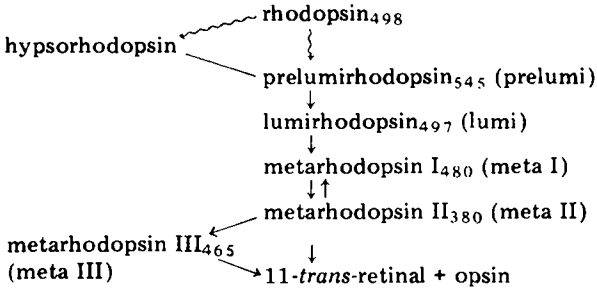
rized in the following reaction cascade [1—6]. In brackets the abbreviation used throughout this paper is given:



The kinetics of the various intermediates have been determined under a variety of conditions. On the one hand it is reported that with sonicated rod outer segments the meta I—II reaction is a first-order process [7—10]. On the other hand there are many references to the fact that the decay kinetics of prelumi, lumi, meta I as well as the formation of meta II are not first-order, but are consistent with a set of parallel, independent first-order reactions [11—15].

Stewart et al. [11—13] found for detergent-solubilized phospholipid-free rhodopsin as well as in solutions containing sonicated or detergent-solubilized rod outer segments that for the lumi-meta I and the meta I—II reactions the absorbance against time could be fitted to the sum of two exponential decay functions. These authors suggested that the reason for this behaviour could be described by assuming two forms of rhodopsin, and associating these with two hypothetical conformations of the protein opsin.

Finally, Hagin's study [14] of the meta I—II reaction in the intact rabbit eye, evaluated by Stewart et al. [12] and Cone's statements [15] on the lumi decay in the frog retina (see also Discussion) indicates that in each case the respective reaction could be described by two independent first-order reactions.

In order to clarify this puzzling point we measured the kinetic occurrence of meta I and meta II under various conditions but allowed the rhodopsin molecule in all experiments to be in its natural environment, the disc membrane.

Materials and Methods

All the experimental conditions of the studies described in this paper are the same as described previously [16,17]. Temperature and pH were measured before and after each measurement in the measuring cell. The measurements of the lumi—meta I and the meta I—II transition were performed on one sample at the wavelengths 417 and 380 nm, respectively.

A typical meta I—II signal together with the evaluation method used is shown in Fig. 1. It should be noted that the ability to observe two components

is strongly connected with the signal to noise ratio. For example, if we assume the signal amplitude A^s (see Fig. 1b) to be 20% of the total signal amplitude then it is not seen for signals with signal^{tot} divided by noise^{tot} less than 5. To obtain an accurate value for the slow component it is necessary to have a precise value for the signal at long times, at least until five times the half time for the slow component is reached. Because the decay of meta II is orders of magnitude slower than its occurrence, it is possible to obtain this asymptote. All signals discussed in this paper correspond in quality to the one in Fig. 1a.

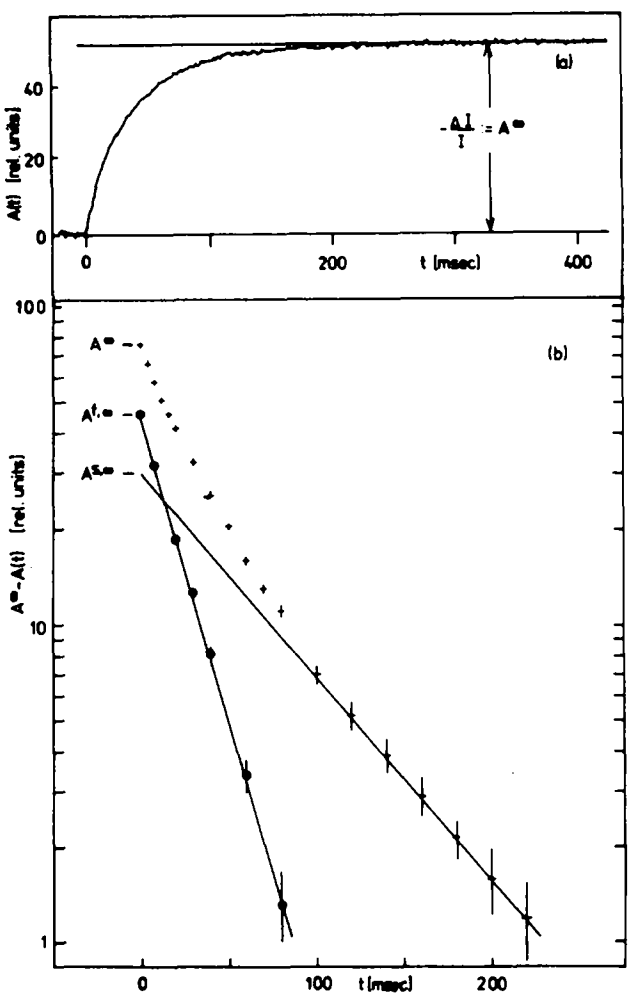


Fig. 1. Part a shows a typical kinetic signal for the meta II occurrence. The total signal amplitude is defined as A^{∞}_{metaII}. Rod outer segments with destroyed outer plasma membrane in 290 mosmol KCl, buffered with 15 mmol PIPES (piperazinediethane sulfonic acid), pH 7.15, temperature 18°C, flash at $t = 0$, wavelength for the measuring beam 380 ± 5 nm. Part b shows the evaluation method used. Crosses are transferred data points from (a) with $A^{\infty}_{metaII} = 76 \pm 2$ relative units. Extrapolation of the linear portion of this function yields the slow component amplitude $A^{s,\infty}_{metaII} = 30 \pm 4$ with $t^s_{1/2} = \ln 2/k^s = 48 \pm 5$ ms. Subtraction of the data with the corresponding extrapolated ones yields the fast component amplitude (indicated with filled circles) $A^{f,\infty}_{metaII} = 46 \pm 6$ and $t^f_{1/2} = \ln 2/k^f = 15 \pm 2$ ms.

Results

Meta I—II transition

For ten temperatures in the range 1—37° C and various pH values the meta I—II transition was studied. For all conditions applied the signals could be fitted to two exponential decay curves as shown in Fig. 1b. Typical results for the pH dependence of the fast and slow component amplitude at some (not all measured results are shown) temperatures are shown in Fig. 2. The corresponding rate constants k^f and k^s are figured in form of an Arrhenius plot in Fig. 3.

Although some of the Arrhenius plots display a significant non-linearity

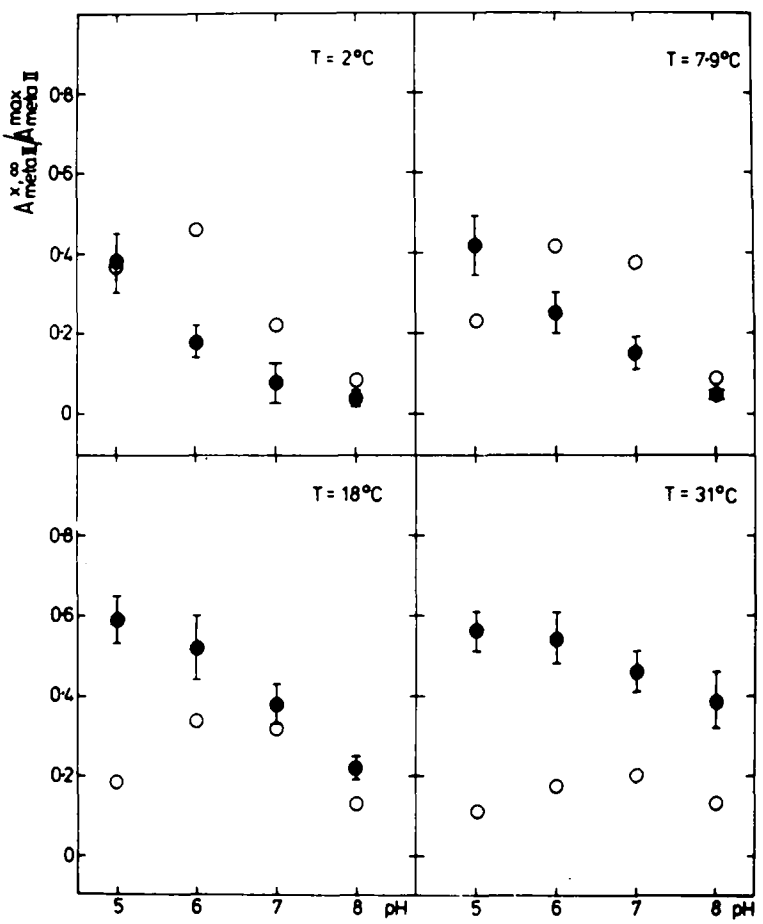


Fig. 2. pH dependence for the normalized fast (filled circles) and slow (open circles) component amplitude of meta II at some temperatures. Results at temperatures 5, 11, 15, 22, 25.5, and 37.5° C (the last temperature only for pH 7) display intermediate situations to the shown pictures. Normalization is in relation to an inner standard, $A^{max,\infty}_{metaII}$, which is the maximal observed total meta II signal amplitude (always obtained at pH 6 and 22° C), to account for differences in concentration for the various preparations. The points are the mean value of at least two measurements. Standard deviation, shown for the fast component, is similar for the slow component amplitude. Evaluation method and nomenclature as described in the legend to Fig. 1.

for temperatures lower 5°C and higher 22°C, we fitted in the temperature midrange (5—22°C) the respective values for the rate constants to a straight line according to $k(T) = A \exp(-E_a/RT)$ (where k denotes the rate constant, E_a the activation energy, R the gas constant, and T the absolute temperature).

At $T = 20°C$ and pH 7 the meta I—II reaction was measured to observe the dependence on the wavelength of the measuring beam. Both the maximal absorbance value and the isosbestic point between meta II and rhodopsin are the same for $A^{f,\infty}_{meta\,II}$ and $A^{s,\infty}_{meta\,II}$ (386 ± 4 and 430 ± 5 nm, respectively).

Lumi—meta I transition

In order to be certain that measurements of the appearance of meta I are

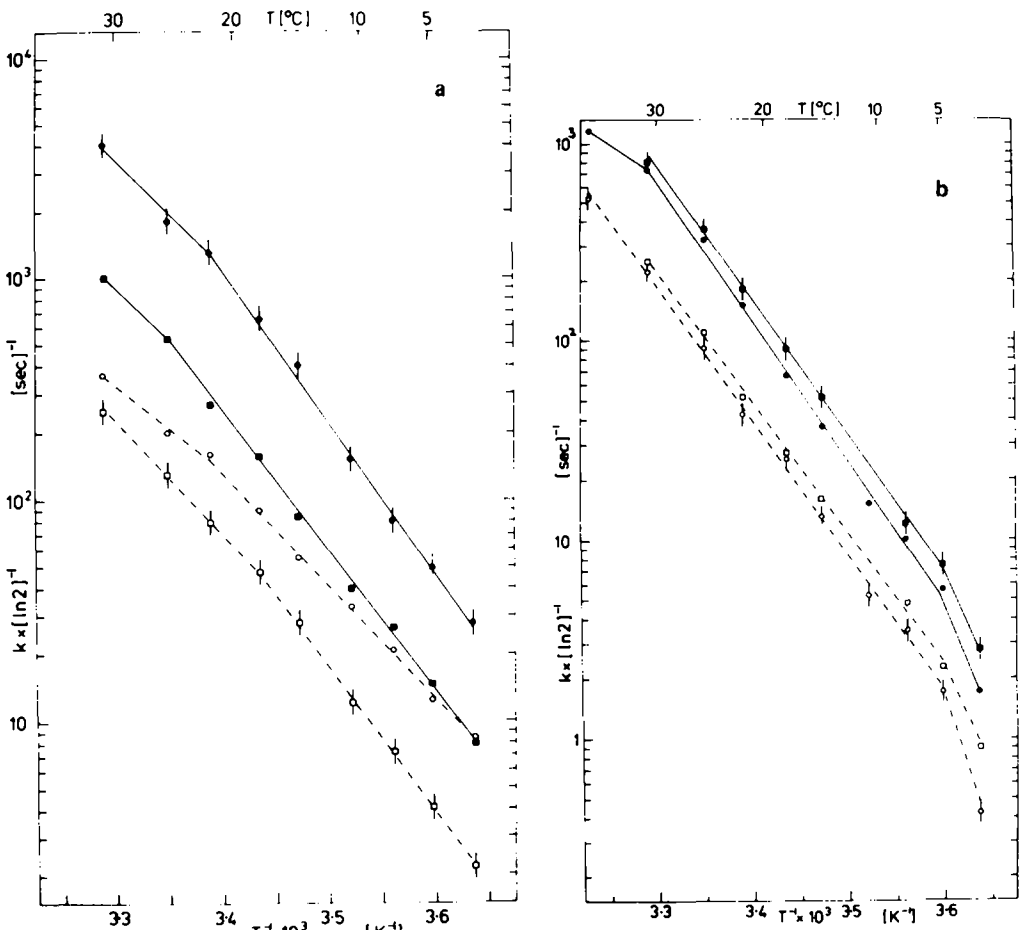


Fig. 3. Arrhenius plot for fast (k^f) and slow (k^s) rate constant of the meta I—II reaction for various pH values. The values for the rate constants are the mean value of at least two measurements. Standard deviation, shown for k^f at pH 5 and 8, k^s at pH 6 and 7, is similar for the other points. The drawn line is the calculated one in the temperature range 5—22°C, from which the activation energy has been calculated. Deviations from this straight line are discussed in the text. (a) ●———●, k^f (E_a = 27 ± 2 kcal/mol); ○———○, k^s (E_a = 21 ± 2), pH 5; ■———■, k^f (E_a = 31 ± 2); □———□, k^s (E_a = 26 ± 2), pH 6. (b) ●———●, k^f (E_a = 34 ± 2); ○———○, k^s (E_a = 31 ± 2), pH 7; ■———■, k^f (E_a = 32 ± 2); □———□, k^s (E_a = 29 ± 2), pH 8.

TABLE I

pH DEPENDENCE FOR THE "f":"s" AMPLITUDE RATIO FOR META I APPEARANCE IN COMPARISON WITH THE ONE FOR META II APPEARANCE AT $T = 2°C$.

(‡) shows once more the values for this temperature obtained in the van't Hoff plot in Fig. 4. In the last two rows the pH dependence for the half time for both the "f" and "s" component of the lumi—meta I reaction is given.

	pH						
	4.5	5.0	5.5	6.0	6.5	7.0	7.4
$A^{f,\infty}_{meta\,I}/A^{s,\infty}_{meta\,I}$	1.8 ± 0.2	1.2 ± 0.2	0.38 ± 0.1		0.4 ± 0.1	0.32 ± 0.1	0.31 ± 0.1
$A^{f,1/2}_{meta\,II}/A^{s,\infty}_{meta\,II}$	1.7		0.36		0.30	0.4	
$A^{f,\infty}_{meta\,II}/A^{s,\infty}_{meta\,II}$ (‡)		1.15		0.4		0.3	
$t^{f}_{1/2}$ (ms)	12	11	7.5		4.5	4.0	3.2
$t^{s}_{1/2}$ (ms)	120	110	22		19.5	14	15

uneffected by the subsequent meta I—II transition, the wavelength of the measuring beam must be at the isosbestic point between meta I and meta II. To find this wavelength the time scale of the kinetic signal was selected to observe the meta I—II reaction, then the wavelength of the measuring beam was decreased from 430 nm until no negative contribution to the kinetic signal was obtained. This was observed at 417 ± 2 nm (for wavelengths above the isosbestic point between meta I and meta II the meta I—II transition is indicated by absorption decrease, corresponding to a negative signal amplitude in our nomenclature).

The evaluation method of the signals for the lumi—meta I transition at 2°C and the various pH values was the same as described in Fig. 1b with the exception that the fast, not time resolved rhodopsin—lumi jump was neglected (this reaction produces an increase in absorption which is in the same direction as the following meta I appearance).

For each sample the lumi-meta I and meta I—II reaction was measured. The results are summarized in Table I.

Discussion

Meta I—II transition

One point which has been overlooked in the literature in comparing activation parameters for the meta I—II reaction is the slope change of the Arrhenius plots in varying the pH. As seen from our results (cf. Fig. 3a and 3b) there is a marked dependence on the activation energy for this reaction. Interestingly, there seems to be some parallel behaviour between the rate constants and the activation energy: If one compares for one component (for example the fast one) at a fixed temperature (for example 22°C) the change of the rate constant with the one for the activation energy in varying the pH from 5 to 7, the rate constant k^f decrease (from 905 s^{-1} at pH 5, 190 s^{-1} at pH 6, to 105 s^{-1} at pH 7) as the activation energy increase (27 kcal/mol at pH 5, 31 kcal/mol at pH 6, and 34 kcal/mol at pH 7), whereas the rate constant at pH 8 with

regard to pH 7 increase again (125 s^{-1}) while the activation energy decreases (32 kcal/mol at pH 8).

Our values for the activation energy at pH 7 are in reasonable agreement with Pratt et al. [18]. He found for this reaction (measured between 10 and 20°C), which was as well described by two first-order reactions, an activation energy of 35 kcal/mol for the fast component and 37 kcal/mol for the slow one. Other literature values are 35 [9] and 33.7 kcal/mol [8] (although only one first-order reaction has been reported there).

Furthermore, the curves in the Arrhenius plots in Fig. 3 are only in the first approximation a straight line, and at a given temperature, the activation energy derived from the slope of the graph is also pH dependent. For the meta I—II transition (cf. Fig. 3) one can see a systematic (i.e. for all pH values) convex curvature.

DSC (differential scanning calorimetry) measurements [19] indicate that the lipid system interferes with the meta I—II transition, because meta II appearance [2] coincides with the start of the phase transition at about −20°C within the rod outer segment lipid system. Since enzyme kinetics are influenced by lipid fluidity [20] we account for the convex curvature in Fig. 3 by the increase of lipid fluidity with increasing temperature [21,22] within the rod outer segments which is associated with the lowering of the activation energy, as indicated by the decrease of the slope in the Arrhenius plot.

Perhaps this is also the reason for the differences found in the literature concerning the activation energy for this reaction [23]: When the thermodynamic parameters are calculated for intervals at lower temperatures, large values for the activation energy and therefore also large positive ones for the activation entropy are found, whereas at higher temperatures smaller values have been claimed. These results agree with our findings that the activation energy decreases with increasing temperature.

Lumi—meta I transition

The isosbestic point between meta I and meta II determined from our kinetic measurements (417 nm) is in good agreement with static measurements [2].

The fast component : slow component amplitude ratio both for the lumi—meta I and the meta I—II transition shows a good agreement at the respective pH values (Table I). This is consistent with the results by Stewart et al. [13] who showed for phospholipid-free rhodopsin solubilized in digitonin that the kinetics of decay of lumi were consistent with double exponential kinetics with approximately the same relative amplitudes as those determined from the meta I decay kinetics. Furthermore, Cone [15] observed also double exponential decay kinetics for the lumi in the frog retina.

In contradiction to the statement that all transitions up to meta I display only intramolecular events, we observe for our preparation a strong dependence of the rate constant, for both "f" and "s" component, on the pH at the given temperature for the lumi—meta I reaction (Table I). This dependence is in opposite direction to the one observed for the meta I—II reaction (compare with Fig. 3).

Equilibrium between two rhodopsin states

Let us now try to describe the temperature- and pH-dependent behaviour of the "f" and "s" component as an equilibrium. If there is such an equilibrium, the ratio of the two amplitudes A^f and A^s, determined from both the lumi—meta I and the meta I—II transitions should be equal to the equilibrium constant for these two forms and should give a straight line in a van't Hoff plot. This is summarized in Fig. 4. The large error bars are due to the uncertainty in determining both A^{tot} and especially A^s. Let δ be the error of A^{tot}, Δ the error in A^s, then the upper and lower limits of the error bars correspond to $(A^f \pm (\Delta + \delta))/(A^s \mp (\Delta + \delta))$.

Also given are some of the results of Stewart et al. [13]. Digitonin-solubilized rhodopsin containing less than 0.2 mol phospholipid per mol rhodopsin and rod outer segments solubilized in cetyl trimethyl ammonium bromide or Emulphogene BC 720 (not shown in the diagram) show approximately the

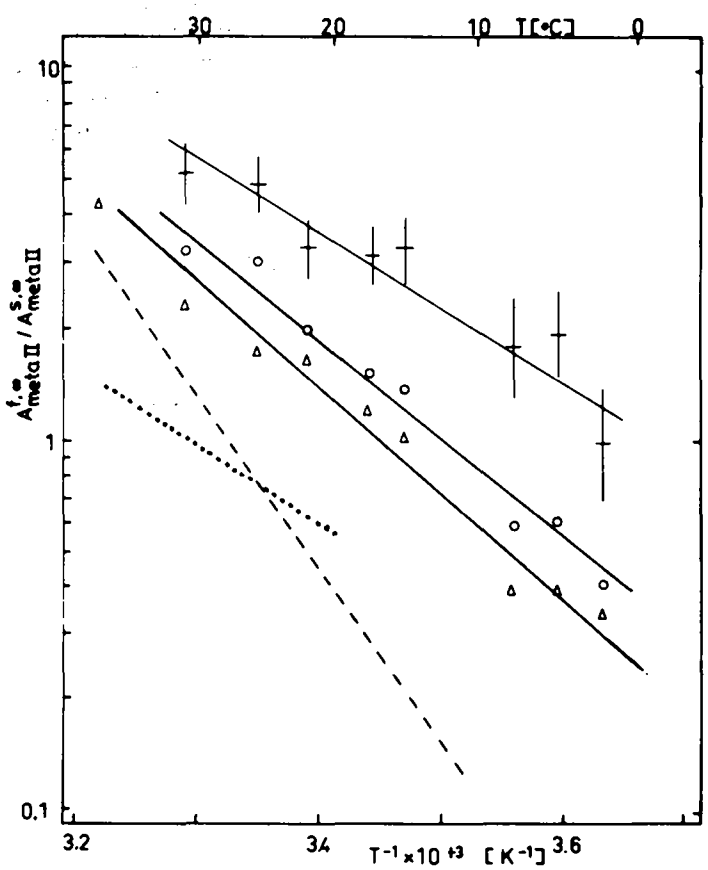


Fig. 4. Van't Hoff plot for the assumed equilibrium between two opsin conformeric states. † †, pH 5 (with error bars), ΔH = 9.1 ± 2 kcal/mol; ○○, pH 6, ΔH = 11.2 ± 2 kcal/mol and △△, pH 7, ΔH = 13.4 ± 2 kcal/mol. Approximately the same error bars for these two pH values as for the first one. As comparison two curves from Stewart et al. [13] are also given: · · · · · ·, phospholipid-free, digitonin-solubilized rhodopsin, pH 6, ΔH = 9.8 kcal/mol; - - - - - -, sonicated rod outer segments in 66% glycerol, pH 6.5, ΔH = 21.9 kcal/mol.

same reaction enthalphy (9.8—13.5 kcal/mol), but that of sonicated rod outer segments (pH 6.5) is much larger (21.9 kcal/mol). Because these authors used in this measurement, which is the most comparable one in view of the rhodopsin state, a solution containing 67% glycerol it is unfortunately not possible, to relate this reaction enthalpy with our calculated values (it is known that glycerol influences the meta I—II equilibrium [2]).

The order of magnitude found for this reaction enthalphy corresponds to those found for the conformational changes of opsin during the meta I—II equilibrium (ref. 2, see also next section) and for transitions between some conformational sub-states of another protein, α-chymotrypsin [24].

It is therefore likely that once more an opsin conformational change could account for the two forms "f" and "s". This is also confirmed by the fact that the observed equilibrium is influenced by pH.

As yet we have nothing said about the point within the photolysis sequence of rhodopsin when this equilibrium is established. Whether or not prelumi displays the same type of double exponential decay kinetics at room temperature has yet to be shown. With certainty we can only state that this equilibrium has to be established before lumi decay. The conclusion from this would be that the time constant for establishing this equilibrium must be of the same order as the prelumi—lumi reaction time constant. This one has been shown to be about 40 ns at room temperature [4]. Such a fast conformational change is not very likely and a plausible assumption is that rhodopsin itself, before bleaching, exists in this proposed equilibrium.

Once more it should be pointed out that from Hagins' study [14] on the intact rabbit eye and from Cone's measurements [15] on the frog retina, there was also found a double exponential kinetic form of meta II occurrence and decay of lumi, respectively. Therefore the observed equilibrium may be due to physiological conditions. As pointed out by Stewart et al. [13] heterogeneity due to the lipid system can also not account for the double exponential kinetics.

Under physiological conditions one can see from Fig. 4 that the rhodopsin^f species is dominant. The physiological consequence of this behaviour is still not known, but the application of this result is able to explain the difficulties in interpretation of some measurements reported in the literature.

Reaction enthalpy for both the meta I^f—II^f and meta I^s—II^s equilibrium

With the results as yet established it is possible to calculate, from the temperature dependence of $A^{tot,\infty}_{metaII}$ together with the known A^f/A^s ratio, the two equilibrium constants

$K^f = A^{f,\infty}_{metaII}/A^{f,\infty}_{metaI}$ and $K^s = A^{s,\infty}_{metaII}/A^{s,\infty}_{metaI}$.

As can be seen from Fig. 5 the reaction enthalpy for both reactions is the same (16 kcal/mol) and in good accordance with the overall one observed by Matthews et al. [2] (the difference may be due to the fact that these authors used digitonin-solubilized rhodopsin). The reaction enthalpy shows a slight pH dependence which is approximately the same for both meta II^f and meta II^s.

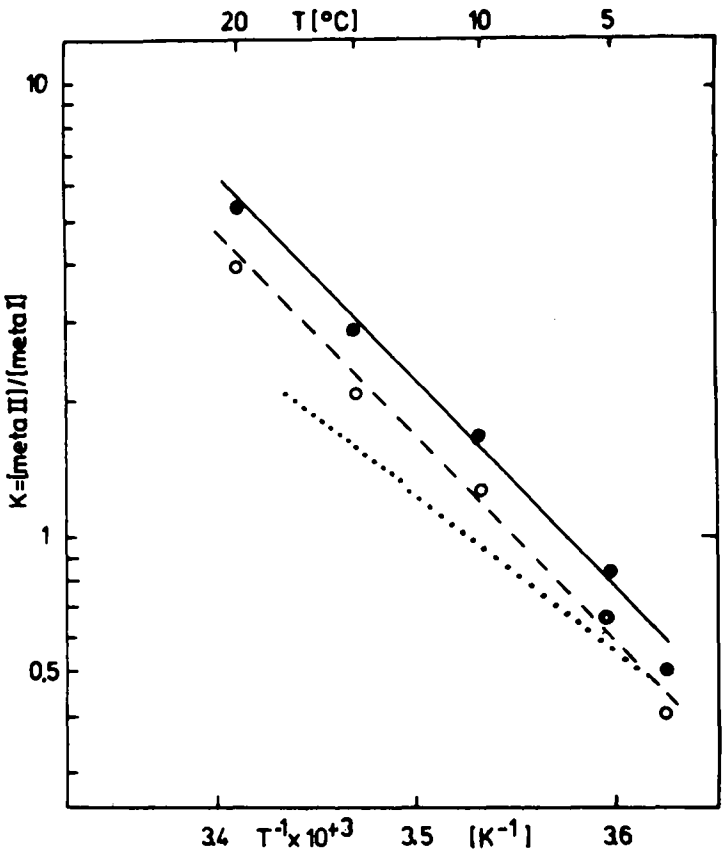


Fig. 5. Van't Hoff plot for both the equilibrium reactions meta $II^f \rightleftharpoons$ meta I^f (●———●) and meta $II^s \rightleftharpoons$ meta I^s (○———○), ΔH = 16 kcal/mol for each "f" and "s", for pH 7. Overall equilibrium reaction from static measurements obtained by Matthews et al. [2]. · · · · · ·, pH 7, digitonin-solubilized rhodopsin, ΔH = 13.1 kcal/mol.

Two rotational relaxation times for rhodopsin

We already noticed that Cone [15] found a double exponential decay of lumi. He pointed out that he was in this case not able to fit his decay of linear dichroism, due to the rotation of rhodopsin, with one relaxation time. Quotation: "However, it does not account for the two stage decay '(of lumi)' observed in many preparations, suggesting that under certain, as yet undefined, conditions the recordings may distinguish two relaxation processes". The measurements made by Cone were carried out at various temperatures. Having in mind that there is an equilibrium between the two rhodopsin states and consequently one of these two species is decreased to such a degree that it is not seen in the kinetic recordings, due to the given signal to noise ratio, could be an explanation why Cone could not find in all recordings a two stage decay both for lumi and linear dichroism. In addition, the requirement of Cone for two rotational relaxation times in the case of the two stage decay of lumi is a strong evidence for a conformationally altered opsin. Because if one assumes that the two rotational relaxation times are only due to heterogeneity in the

lipid system, it is difficult to explain, why there are not two relaxation times required in the case of the one stage lumi decay recordings.

Photoreversibility of meta I and meta II (Williams et al. [25,26])

It was shown that there is a pH- and temperature-dependent equilibrium between two meta I states: one (meta I) which can photoreversed to rhodopsin and the other not (meta I'). The reaction enthalpy for this supposed equilibrium meta I $\rightleftharpoons$ meta I' has been shown to be 11.3 kcal/mol. If one compares this value with the results we obtained, it is possible to equate meta I with our meta I^s and meta I' with our meta I^f. The consequence would therefore be that meta I^f cannot be photoreversed whereas meta I^s should be photoreversible.

Furthermore, also for the meta II, there was found a distinct behaviour in irradiating it: some meta II becomes photoinduced meta III and another fraction slowly becomes rhodopsin. Once more there is a difference in the behaviour of one and the same spectroscopic product. Together with the conclusions of the next section this behaviour could perhaps be interpreted by the assumption that meta II^f can become photoinduced meta III, whereas meta II^s is slowly becoming rhodopsin after irradiation.

Slow photoproducts beyond meta II

Baumann [6] showed that meta II reacts by two pathways before hydrolysis: one in which meta III is involved and the other a direct path. As will be described in detail in another paper we have shown that for a given temperature (10°C) and predicted from the van't Hoff plot in Fig. 4 there is the same pH dependence for these meta II molecules, which react via meta III, as for those, which are in the meta II^f state. Therefore all meta II^f molecules react via meta III before hydrolysis, whereas all meta II^s molecules decay directly to retinal and opsin.

Acknowledgements

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