STUDIES ON THE METASTABLE STATES IN THE RHODOPSIN CYCLE

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SUMMARY

Photic reaction of rhodopsin in the range of —195° to 20° has been examined by means of measurement of optical density at room temperature after illumination or re-illumination at low temperature. The experimental results are as follows:

1. The amount of rhodopsin remaining after illumination is constant in two regions of temperature (—195° to —140° and —80° to —20°).

2. When illuminated previously at —40°, the amount of rhodopsin is increased by re-illumination below —100°, presenting almost a constant value below —140°.

3. When rhodopsin solution illuminated at any temperature below —140° is re-illuminated at —195°, the amount of rhodopsin is identical with that of the non-re-illuminated; in the range above —100°, the former becomes more abundant than the latter.

On the basis of the experimental results described above, we have discussed the photochemical properties of the labile fraction of lumi- or meta-rhodopsin and the essential of the "liquid air illuminated rhodopsin".

INTRODUCTION

In previous work[1] it was found that a rhodopsin solution illuminated sufficiently in dry ice–acetone or liquid air still retained respectively 50–60 % or 60–80 % rhodopsin, as shown by measurement of 500 mμ absorption (difference) at room temperature. Furthermore, when a solution illuminated in dry ice–acetone was re-illuminated in liquid air, the amount of rhodopsin became more abundant than when illuminated in dry ice–acetone alone. (This phenomenon we called "photo-recovery"[2].) These facts appear to suggest that some metastable state such as being stable in liquid air is involved in the rhodopsin cycle. In order to confirm the existence of this metastable state, the photic reaction of rhodopsin in the range of —195° to 20° was investigated in detail.

METHODS

Two unsilvered Dewars of different sizes were combined for controlling the temperature of the rhodopsin solution under illumination (Fig. 1). According to the temperature, the outer Dewar was filled either with liquid nitrogen (—195° to —78°) or with

water (—70° to 20°). A liquid filling the inside Dewar was chosen from the following series: Liquid nitrogen for —195°, liquid air for —186°, EPA-mixture (ether, isopentane and alcohol in volume ratio 8:3:5) for —170° to —80°, alcohol for —78° to 0° and water for 20°. Temperature of the inner liquid—regarded as that of the sample—was monitored with a thermometer (platinum resistance thermometer for —195° to —70°, toluene thermometer for —80° to 20°) within the error of ± 1°.

All the experiments were carried out under dim red light. Cattle rhodopsin solution was prepared by the same method as described in the previous paper[1]. Every solution was adjusted to pH 9.2 by addition of 1/20 *M* borax. Optical purity ($E_{400\ m\mu}/E_{500\ m\mu}$) : 0.3–0.5, optical density at 500 mμ : 0.25–0.4.

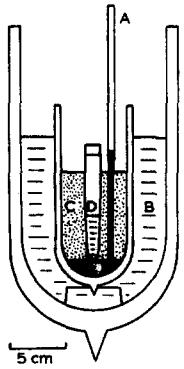

Fig. 1. Diagram of an apparatus for controlling temperature during illumination. A, thermometer; B, liquid nitrogen or water; C, liquid nitrogen, liquid air, EPA-mixture, alcohol or water; D, rhodopsin solution; E, mercury.

Before every experiment, a centrifuge tube containing 3 ml rhodopsin solution was immersed in liquid nitrogen and then placed in the inside Dewar with constant temperature. After being kept herein for more than 10 min*, the sample was laterally illuminated for 5 min by a 250 watt incandescent lamp (8,000 lux)**. Immediately after illumination, the sample was transferred into liquid nitrogen*** and, when

* In a preliminary experiment, it was established that when the sample, which had been previously kept in liquid nitrogen, was immersed in alcohol of —20° it took about 5 min to reach a temperature equilibrium.

** In order to determine the suitable condition of illumination, we compared 8,000 lux × 5 min illumination with 8,000 lux × 10 min illumination with reference to the relative amount of residual rhodopsin measured at room temperature. As shown in the table, 8,000 lux × 5 min illumination and 8,000 lux × 10 min illumination resulted in almost equal values for every temperature during illumination. Therefore, we adopted 8,000 lux × 5 min as the condition of sufficient illumination in the present experiment.

TABLE I

Exposure time	*Temperature during illumination*		
	— 195°	*— 150°*	*— 100°*
5 min	82.7 %	80.5 %	61.8 %
10 min	82.1 %	80.3 %	59.6 %

*** A rhodopsin solution illuminated in dry ice–acetone showed no further change in the absorption spectrum, even though it was afterwards cooled to temperature of liquid nitrogen in the dark[2]. This fact indicates that liquid nitrogen is a suitable medium for the conservation of the sample.

necessary, thawed in a water bath. Thereafter, it was incubated at 23° in darkness for 1 hour. Optical density at 500 mμ was determined at room temperature (23°–28°) by a Beckman type spectrophotometer and the measurement was repeated again after rhodopsin had been completely bleached by illumination for 20 min with an incandescent lamp (8,000 lux). We regarded the difference of optical densities thus obtained as a measure of the amount of rhodopsin contained in the illuminated sample. Referring to the amount of rhodopsin likewise obtained from the dark sample kept in liquid nitrogen, we could calculate the relative amount of rhodopsin in %.

RESULTS

Experiment 1

In order to determine the relation between the amount of rhodopsin and the temperature under illumination, we carried out the following experiment. Rhodopsin solution was divided into a number (four to nine) tubes. One of them was kept at —195° in the dark, while any of the others was illuminated at a certain temperature between —195° and 20°.

The results are summarized in Fig. 2, where the relative amounts of rhodopsin after illumination at various low temperatures were plotted against the temperatures concerned. Fig. 2 indicates a general trend, namely that the amount of rhodopsin decreases as temperature goes up. This decrease, however, proceeds by no means uniformly, but stepwise. It should be noticed that the amount remains almost constant in two different temperature regions (—195° to —140° and —80° to —20°), which can probably be regarded as denoting two different states of rhodopsin illuminated at low temperature.

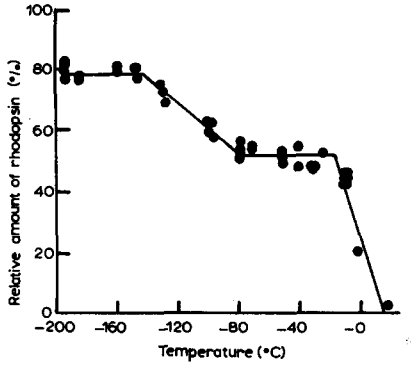

Fig. 2. Relationship between amount of rhodopsin and temperature of illumination.

Experiment 2

Assuming that Fig. 2 is suggestive of two different states in the course of the bleaching process of rhodopsin, one would be inclined to consider the "photo-recovery" as nothing but the transition from one state (caused by illumination of rhodopsin at —80° to —20°) to another (resulting from illumination of rhodopsin at —195° to —140°). Accordingly, one would expect the "photo-recovery" to be clearly revealed by re-illumination in the lower temperature range (—195° to —140°) after illumination in the higher range (—80° to —20°).

(*a*) As the temperature for pre-illumination —40° was adopted and as the temperatures for re-illumination various temperatures below —40°.

We divided the rhodopsin solution into twelve tubes containing 3 ml each. One of them was preserved at —195° in the dark as the basic sample. Five tubes were illuminated at —40° and preserved in liquid nitrogen. They were then re-illuminated at —195°, —161°, —131°, —100° and —70°, respectively. The corresponding five different temperatures and —40° were adopted for single illumination of the remaining six tubes.

The experimental results are shown in Fig. 3. We find that any sample re-illuminated at a temperature below —100° is more abundant in the relative amount of rhodopsin than that illuminated once at —40° (photo-recovery). Furthermore, the amount of rhodopsin in the sample re-illuminated at any temperature lower than —131° is considered as almost constant (68 %), while the sample re-illuminated above —131° denotes a decrease in the amount of rhodopsin in accordance with the rise in temperature for re-illumination, until the re-illumination at —70° seems to become almost ineffective.

This result indicates to us that a considerable increase in the amount of rhodopsin (photo-recovery) is caused by re-illumination in the temperature range between —195° and —140°, where a metastable state suggested by Fig. 2 would be formed on illumination.

On the other hand, every sample re-illuminated at certain temperature from —195° to —100° (solid circle) indicates evidently a lower value in the relative amount of rhodopsin than that illuminated once at the corresponding temperature (open circle). This fact demonstrates that the state of rhodopsin illuminated at —40° cannot always be completely changed by re-illumination to the state formed by single illumination at temperature below —140°.

(*b*) We carried out a further experiment of re-illumination at —195° after illumination at various temperatures, in order to examine the relation of the amount of rhodopsin to the temperature during pre-illumination.

Rhodopsin solution was divided into thirteen tubes containing 3 ml each. One of them was kept at —195° in the dark. The rest were treated as six pairs. Each pair was illuminated at —186°, —162°, —131°, —100°, —70° and —39°, respectively,

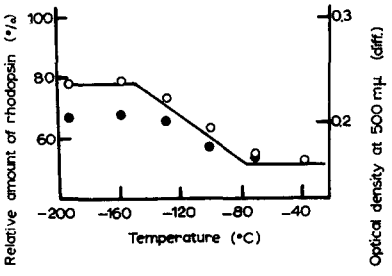

Fig. 3. Relationship between amount of rhodopsin and temperature of re-illumination. Solid circles: re-illuminated at various temperatures after illumination at —40°. Open circles: illuminated once at the corresponding temperatures. Solid line: from Fig. 2.

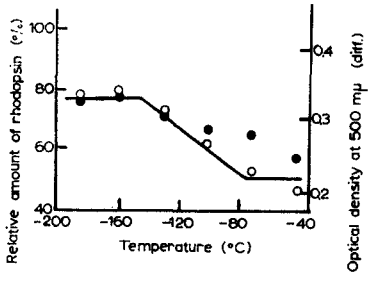

Fig. 4. Relationship between amount of rhodopsin and temperature of pre-illumination. Solid circles: re-illuminated at —195° after illumination at various temperatures. Open circles: illuminated once at the corresponding temperatures. Solid line: from Fig. 2.

and then preserved in liquid nitrogen. One of each pair was subsequently re-illuminated at—195°.

As Fig. 4 shows, below —131° scarcely any difference is found between amount of rhodopsin in the re-illuminated sample (solid circle) and in the control (open circle); above —100°, however, the former becomes obviously more abundant than the latter.

An increase in the amount of rhodopsin (photo-recovery) was most clearly revealed by the samples pre-illuminated at —39° and —70°. This fact suggests to us that illumination in the temperature region between —80° and —20° would produce one state of rhodopsin with reference to the "photo-recovery". Illumination in the temperature region between —195° and —140° is considered as producing another state of rhodopsin, on the basis of the fact that the re-illumination at —195° is ineffective.

DISCUSSION

From the spectroscopic study of rhodopsin at low temperature, WALD, DURELL AND ST. GEORGE[3] found that two different states of rhodopsin, named lumi- and meta-rhodopsin, were demonstrable in the decomposing process at low temperature. Lumi- and meta-rhodopsin are considered to correspond to our rhodopsin illuminated at —70° and at —20°, respectively. However, we obtained the experimental result that when a rhodopsin solution is illuminated at any temperature in the range of —80° and —20° almost a constant value for the relative amount of rhodopsin is obtained. (Fig. 2). We also established in the previous work[4] that lumi-rhodopsin could not be distinguished from meta-rhodopsin with reference to the amount of photo-recovered rhodopsin. Therefore, lumi-rhodopsin can be considered to be identical with meta-rhodopsin so far as our experiments are concerned, and both of them may be involved in a single state of rhodopsin illuminated at any temperature from —80° to —20°.

HUBBARD AND KROPF[5] considered that lumi- or meta-rhodopsin is a steady-state mixture, which is composed of rhodopsin, iso-rhodopsin and such labile fractions as are stable below —15°, and that this labile fraction is regenerated to rhodopsin and iso-rhodopsin by the action of light. If we accept their opinion, the fact that the amount of rhodopsin was independent of the temperature of illumination in the range —80° to —20° must be explained on the assumption that every rhodopsin solution illuminated at any temperature between —80° and —20° (lumi- or meta-rhodopsin) contains an equal amount of the labile fraction in this range of temperature. The labile fraction produced in the range of —100° and —40° must be converted into rhodopsin (or iso-rhodopsin) by re-illumination at —195°, since we could observe the increase in the relative amount of rhodopsin (Fig. 4). This fact probably supports the view of HUBBARD AND KROPF that the labile fraction is regenerated to rhodopsin and iso-rhodopsin by the action of light.

We, on the other hand, found the following: (1) the relative amount of rhodopsin was independent of the temperature of illumination in the range —195° to—140° (Fig. 2); (2) when the solution previously illuminated at —40° was re-illuminated below —140°, the amount of rhodopsin increased towards a constant value (68%) (Fig. 3); (3) with a solution illuminated below —140° there was no photo-recovery on re-illumination at —195° (Fig. 4). In view of these results, the temperature —140°

attracts our attention with regard to thermal stabilization of the metastable state formed in the photo-decomposing process of rhodopsin. The "liquid air illuminated rhodopsin" which was proposed in a previous paper[2] may be considered as a short-lived state, being stable below —140°, and lying between rhodopsin and lumi-rhodopsin in the rhodopsin cycle. "Liquid air illuminated rhodopsin" is considered to be partly changed to the labile fraction (component of lumi- or meta-rhodopsin) in the dark by warming it to approx. —40°. On the other hand, lumi- or meta-rhodopsin may not be converted to "liquid air illuminated rhodopsin" when cooled to —195° in the dark[2]; the change would occur only on exposure at —195°.

We cannot yet decide at present whether "liquid air illuminated rhodopsin" is a single intermediate or a steady-state mixture. On the basis of the view that lumi- or meta-rhodopsin is a steady-state mixture, "liquid air illuminated rhodopsin" may also be regarded as another steady-state mixture, differing from lumi- or meta-rhodopsin in the property of the labile fraction. In the field of radiochemistry, some free radicals are found to be stable below —140°[6,7]. This suggests that "liquid air illuminated rhodopsin" may be a kind of free radical. Our future research will be conducted along these lines.

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REFERENCES

1 T. YOSHIZAWA AND Y. KITÔ, *Ann. Rep. Sci. Works, Fac. Sci. Osaka Univ.*, 6 (1958) 27.
2 T. YOSHIZAWA AND Y. KITÔ, *Nature*, 182 (1958) 1604.
3 G. WALD, J. DURELL AND R. C. C. ST. GEORGE, *Science*, 111 (1950) 179.
4 Y. KITÔ AND T. YOSHIZAWA, *Annotationes Zool. Japon.*, 33 (1960) 7.
5 R. HUBBARD AND A. KROPF, *Proc. Natl. Acad. Sci., U.S.*, 44 (1958) 130.
6 M. S. MATHESON AND B. SMALLER, *J. Chem. Phys.*, 23 (1955) 521.
7 J. A. CHORMLEY AND A. C. STEWART, *J. Am. Chem. Soc.*, 78 (1956) 2934.