Biochimica et Biophysica Acta, 325 (1973) 323–331

BBA 46638

KINETICS OF REACTION OF THE SULFHYDRYL GROUPS OF RHODOPSIN

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(Received June 4th, 1973)

SUMMARY

The kinetics of reaction of the sulfhydryl groups of rhodopsin have been investigated using the sulfhydryl reagent 4-4′-dithiodipyridine. The spectral changes associated with the sulfhydryl group reaction and the photo-initiated thermal intermediates were monitored simultaneously.

In the unbleached digitonin-extracted rhodopsin two separate sulfhydryl group reaction rates which varied somewhat as a function of pH were observed from pH 5.0–8.0. Approximately two sulfhydryl groups reacted with reagent with first-order half-lives of 24 min and 144 min at pH 7.5, 3 °C.

Upon illumination two additional sulfhydryl groups were exposed with a single first-order half-life of 66 min at pH 7.5, 3 °C. The single rate varied little as a function of pH from pH 5.5–7.5. The following data indicated a correspondence between the thermal decay of metarhodopsin II_{380} and the light-induced sulfhydryl group exposure: comparison of the kinetics of the sulfhydryl exposure and the thermal decay of rhodopsin photoproducts; the constancy of reaction rate as a function of pH; the correspondence of Q_{10} values; and the kinetics of the sulfhydryl reagent reaction when the reagent was added after illumination at particular stages of the intermediate sequence.

The results suggest that the two sulfhydryl groups titratable in the dark are probably located in different environments in the protein structure and show that the two sulfhydryl groups observed after illumination are exposed simultaneously in the thermal decay of metarhodopsin II_{380}.

INTRODUCTION

The titration of the sulfhydryl groups of proteins have provided useful information on the conformation changes of the proteins and the relation between active sites and cysteine residues. The first sulfhydryl studies of the visual pigment, rhodopsin, indicated that at pH 9.5 sulfhydryl groups were titratable in the dark and that additional titratable sulfhydryl groups were exposed after illumination[1]. A possible relation to transduction was suggested. A crucial aspect of that suggestion was the relationship between the sulfhydryl group exposure and rhodopsin photoproducts. Initial experiments on this relationship isolated metarhodopsin I_{478} at −29 °C (pH 9.5) and showed that the light-induced sulfhydryl groups were not titratable up to that stage[2].

In later experiments the sulfhydryl group reaction was followed with concurrent monitoring of rhodopsin thermal intermediates using silver Tris at pH 7. The results indicated that the sulfhydryl groups were exposed after the formation of metarhodopsin II_{380}[3]. In that case, however, four sulfhydryl groups were titrated in the dark and metarhodopsin II_{380} rather than metarhodopsin I_{478} was the predominant thermal product observed after illumination. Also the reagent apparently reacted with the binding site of the chromophore at the metarhodopsin II_{380} stage. The sulfhydryl results are summarized in Table I.

TABLE I

SUMMARY OF SULFHYDRYL DATA

Ref.	*Solution*	*Method*	*No. dark*	*No. light*	*Added information*
Wald and Brown[1]	Rhodopsin/digitonin, pH 9.5	$Ag(NH_3)_2^+$	2	2	
Erhardt *et al.*[2]	Rhodopsin/digitonin, pH 9.5	$Ag(NH_3)_2^+$	1	3 (+23 °C) 0 (−29 °C)	SH groups not exposed before metarhodopsin I_{478}
Ostroy *et al.*[3]	Rhodopsin/digitonin, pH 7	$Ag(Tris)_2^+$	4	2	SH groups exposed after the formation of metarhodopsin II_{380}
	Rhodopsin/digitonin, pH 7	DTNB*	1–2	2–3	
Heller[4]	Rhodopsin/CTAB**, pH 7	DTNB	0	1	
Kimble and Ostroy (This paper)	Rhodopsin/digitonin, pH 5.0–7.5	4-PDS***	2	2	In dark 2 separate rates. Single rate in light. Light SH exposure coincident with thermal decay of metarhodopsin II_{380}
	pH 8		2	3	

* 5,5′-dithio-bis-(2-nitrobenzoic acid).
** cetyltrimethylammonium bromide.
*** 4,4′-dithiodipyridine.

To avoid the above difficulties the reagent 4-4′-dithiodipyridine was used[5]. It could be used at neutral and other pH values, reacted with only two sulfhydryl groups in the dark, yielded apparently normal intermediates on illumination, and did not appear to react with the chromophore binding site. Also this reagent is known to react rapidly and completely with fully exposed sulfhydryl groups[5]. Moreover, its spectral change at 324 nm could be monitored simultaneously with rhodopsin thermal product changes, thus permitting a kinetic study of its reaction similar to previous studies with hemoglobin[6,7].

MATERIALS AND METHODS

Rhodopsin solutions in 1.4% digitonin, 0.03 M phosphate buffer were prepared

from dark adapted frozen bovine retinas (Hormel Co.) according to the procedure of Erhardt *et al.*[2]. The purity ratios were approx. 400 nm/500 nm=0.4, 278 nm/500 nm=2.5. The solutions were stored in the dark at 3 °C until ready for use and were discarded after 2–3 weeks of age.

The 4,4′-dithiodipyridine reagent was prepared at a concentration of 0.28 mg/ml. Reagent solutions were stored at 3 °C under N_2 and never kept for longer than two days[5,6]. The reaction mixture consisted of 0.4 ml dithiodipyridine solution and 0.6 ml of rhodopsin solution previously diluted with digitonin buffer for a final 500-nm absorbance of approx. 0.5. The solution pH was adjusted in the range pH 6.0–8.0 using 0.1 M HCl or NaOH. For adjustment below pH 6.0 the rhodopsin–phosphate buffer was deionized by passage through a mixed-bed ion exchange column and re-buffered with the appropriate 1 M sodium acetate buffer[2]. The sample was placed in a jacketed 1-cm square cuvette in a Cary 14 recording spectrophotometer. Unless otherwise stated, the experiments were performed at 3 °C and the rhodopsin solution was incubated for approx. 20 h with thiol reagent. The sample was illuminated for 7 min using a 0.5-inch fiber optics illuminator and a Corning 3-70 filter ($\lambda > 500$ nm). Spectra were then taken at appropriate intervals. At the end of the experiments the sample was warmed to 20 °C and 0.02 ml of 2 M hydroxylamine was added. The number of light exposed sulfhydryl groups per mole of rhodopsin were calculated based on the percent bleaching determined from the initial 500-nm absorbance and the absorbance remaining at 500 nm after reaction with hydroxylamine. No compensation was made for the small contribution to the 324-nm reagent peak by the rhodopsin intermediates (<0.1 SH/rhodopsin). An extinction coefficient of 40600 was used for rhodopsin at 500 nm[8]. The extinction coefficients for 4-4′-dithiodipyridine at 324 nm determined by reaction with cysteine, were 22400 (pH 5.0); 21900 (pH 5.5); 22200 (pH 6.0); 21300 (pH 7.0); 20700 (pH 7.5); and 18700 (pH 8.0). The pH variation of the extinction coefficient was similar to previous work with 2,2′-dithiodipyridine[5].

The kinetics were analyzed as single or concurrent first-order processes con-

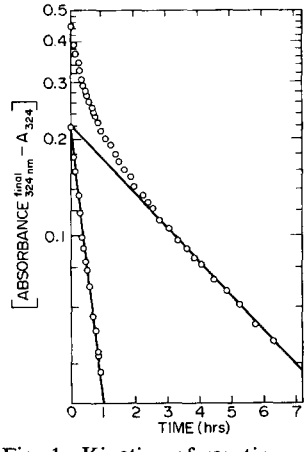

Fig. 1. Kinetics of reaction of the dark sulfhydryl groups at pH 7.5, 3 °C. One sulfhydryl per rhodopsin corresponds to an absorbance change of 0.21. Analyzed as two simultaneous first-order reactions[2].

sistent with previous studies[2,9]. The sample absorbance was taken as the difference between the measured value and the final absorbance and plotted on a log scale *versus* time. In the case of concurrent first-order processes (Fig. 1), the absorbance values of the slowest process were subtracted from the more rapid process and the difference replotted on a log scale. At pH 6 and above, the absorbance changes at 500 nm were used to monitor the thermal decay of metarhodopsin II_{380} (refs 9, 13). At 3 °C the thermal decay of metarhodopsin II_{380} is rate controlling[10] and once the metarhodopsin $I_{478} \rightleftharpoons II_{380}$ equilibrium has been established, the absorbance changes of the former may be used to monitor the thermal decay of metarhodopsin II_{380} (ref. 11). Increasing reagent concentration did not alter reaction kinetics during metarhodopsin II_{380} decay.

RESULTS

A representative experiment illustrating the kinetics of reaction of the dark sulfhydryl groups of rhodopsin is shown in Fig. 1. Two first-order processes with somewhat different rate constants are observed. The stoichiometry and kinetics of the dark sulfhydryl groups at a number of pH values are presented in Table II. The more rapidly reacting sulfhydryl group does not seem to represent one complete group at pH 5–7 but approaches that value at pH 7.5–8.0. Its rate of reaction appears to decrease at increasing pH. The slower reacting group represents a complete group at all pH values and shows the slowest reaction at pH 7.

The spectra illustrating the light-induced photoproduct and sulfhydryl changes are shown in Figs 2–4. A representative kinetic plot for pH 7.5 is shown in Fig. 5 and

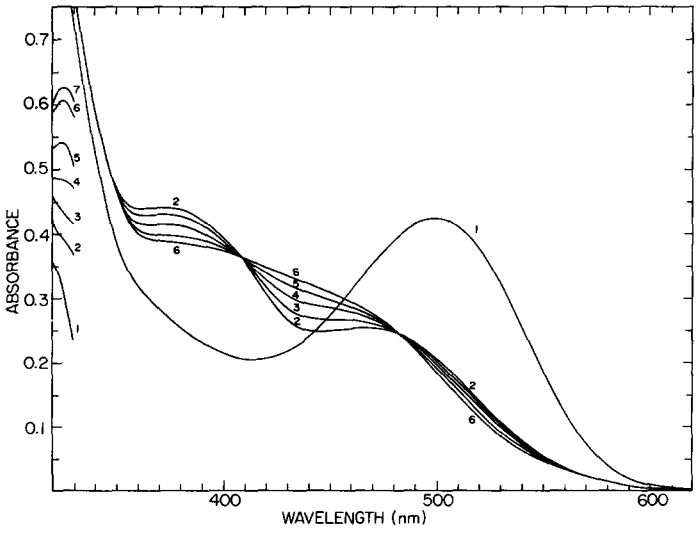

Fig. 2. Spectral changes at pH 5.5, 3 °C. Absorbance in 320–330-nm region reduced by 0.5. Baseline absorbance at 324 nm prior to reaction was 0.48. Sulfhydryl reagent added 19 h before start of illumination. Spectrum rate, 2 nm/sec. Spectrum 1 was started 5 min before the start of the 7-min illumination. Spectrum 2 was started 1 min after the end of the illumination. Spectrum 3, 21 min, Spectrum 4, 56 min, Spectrum 5, 106 min, Spectrum 6, 211 min and Spectrum 7, 466 min after no further reagent changes at 324 nm. Sample was 78% bleached.

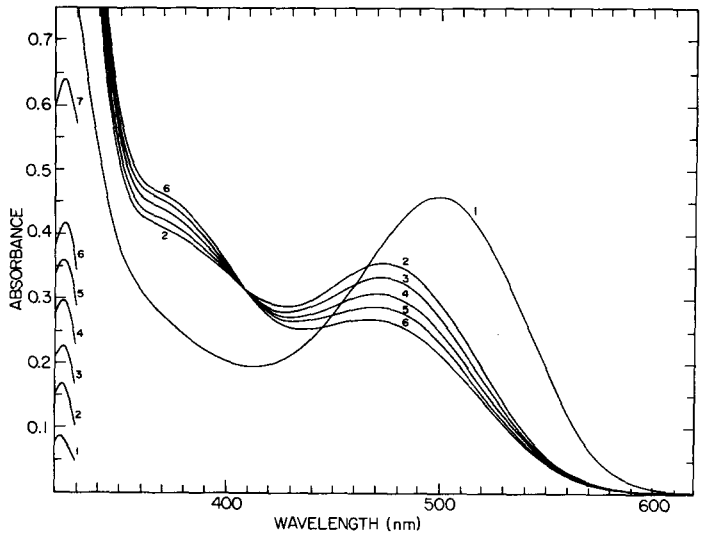

Fig. 3. Spectral changes at pH 7.0, 3 °C. Absorbance in 320–330-nm region reduced 0.5. Baseline absorbance at 324 nm prior to reaction was 0.42. Sulfhydryl reagent added 21 h before start of illumination. Spectrum rate, 2.5 nm/sec. Spectrum 1 was started 4 min before the start of the 7-min illumination. Spectrum 2 was started 0.2 min after the end of the illumination. Spectrum 3. 11.4 min, Spectrum 4, 27 min, Spectrum 5, 53 min, Spectrum 6, 82 min and Spectrum 7, 560 min, Sample was 80% bleached.

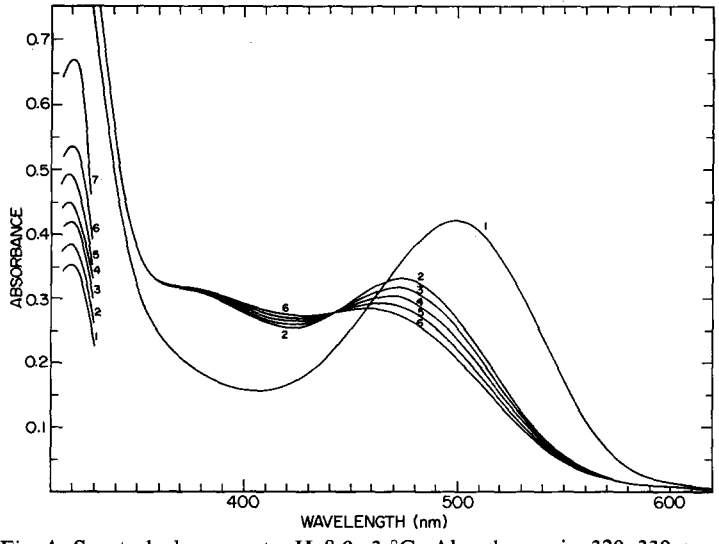

Fig. 4. Spectral changes at pH 8.0, 3 °C. Absorbance in 320–330-nm region reduced by 1.0. Baseline absorbance at 324 nm prior to reaction was 0.76. Sulfhydryl reagent added 5 h before start of illumination, Spectrum rate 2.5 nm/s. Spectrum 1 was started 4 min before the start of the 7-min illumination. Spectrum 2 was started 0.5 min after the end of the illumination. Spectrum 3, 7 min, Spectrum 4, 18 min, Spectrum 5, 23 min, Spectrum 6, 44.5 min and Spectrum 7 478 min. Sample was 80% bleached.

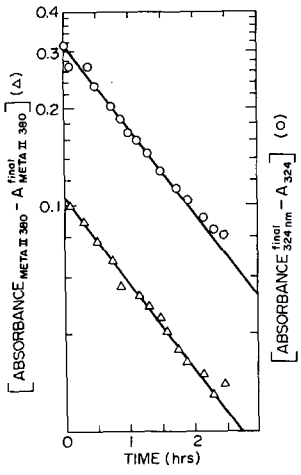

Fig. 5. Kinetics of reaction of light-induced sulfhydryl groups and the thermal decay of metarhodopsin II_{380} at pH 7.5, 3 °C. One sulfhydryl group per rhodopsin bleached represents an absorbance change of 0.17.

the complete stoichiometric and kinetic values are presented in Table III. From pH 5.0–7.5, two sulfhydryl groups are exposed simultaneously on illumination and the rate of exposure of the two sulfhydryl groups is consistent with the thermal decay of metarhodopsin II_{380}. The rate varies little as a function of pH. At pH 8 three sulfhydryl groups are exposed simultaneously and the rate of reaction is more rapid. However, the spectral changes and sulfhydryl group exposure proceed at the same rate.

DISCUSSION

The light induced exposure of sulfhydryl groups

The correspondence in rate between the light-induced exposure of the additional sulfhydryl groups of rhodopsin and the thermal decay of metarhodopsin II_{380} is evidence for coincidence of these processes (Table III). A correlation coefficient of 0.96 (18 experiments) was obtained when comparing the rates of these two processes. As added evidence, the rates of reaction and the number of sulfhydryl groups remain relatively constant as a function of pH (Table III). At lower pH the metarhodopsin $I_{478} \rightleftharpoons II_{380}$ equilibrium favors metarhodopsin II_{380}[9,12] and the reaction is more rapid[9], while the thermal decay of metarhodopsin II_{380} is pH independent[13]. Thus if the light-induced sulfhydryl groups were exposed earlier than the thermal decay of metarhodopsin II_{380}, on both kinetic and concentration grounds one would expect a pH dependence of that exposure.

Additional information on the intermediate state involved in sulfhydryl group exposure was obtained by observing the rate constants as a function of temperature. At pH 7.5, 9 °C, the half-life of reaction of the sulfhydryl group was approx. 0.5 h and the Q_{10} for the sulfhydryl group exposure is therefore 3.5. Under the same conditions, the Q_{10} for the thermal decay of metarhodopsin II_{380} is 3.3. This value compares favorably with previous work[13,14] and is different from other rhodopsin intermediate reactions[10].

TABLE II

DARK SULFHYDRYL GROUPS OF RHODOPSIN

	pH					
	5.0	*5.5*	*6.0*	*7.0*	*7.5*	*8.0*
Stoichiometry						
First group: No. SH/rhodopsin (±S.D.)	0.3 (±0.2)	0.5 (±0.07)	0.5 (±0.1)	0.7 (±0.2)	0.7 (±0.2)	0.9 (±0.3)
Second group: No. SH/rhodopsin (±S.D.)	0.9 (±0.04)	0.9 (±0.01)	1.1 (±0.2)	1.2 (±0.2)	1.1 (±0.1)	1.2 (±0.1)
Kinetics						
First group: half life, 3 °C (h) (±S.D.)	<0.1	<0.1	<0.2	0.2 (±0.05)	0.4 (±0.1)	0.4 (±0.2)
Second group: half life, 3 °C (h) (±S.D.)	2.3 (±0.2)	1.8 (±0.2)	1.7 (±0.6)	2.8 (±0.4)	2.4 (±0.4)	1.0 (±0.2)

TABLE III

LIGHT-EXPOSED SULFHYDRYL GROUPS OF RHODOPSIN

	pH					
	5.0	*5.5*	*6.0*	*7.0*	*7.5*	*8.0*
Stoichiometry						
No. SH/rhodopsin bleached (±S.D.)	1.3 (±0.003)	1.7 (±0.05)	1.9 (±0.1)	2.0 (±0.2)	1.8 (±0.2)	3.1 (±0.4)
Kinetics						
Sulfhydryl groups: half life, 3 °C (h) (±S.D.)	1.4 (±0.2)	1.2 (±0.2)	1.0 (±0.05)	1.3 (±0.2)	1.1 (±0.2)	0.5 (±0.1)
Metarhodopsin II_{380}: half life, 3 °C (h) (±S.D.)	1.1 (±0.1)	1.0 (±0.1)	0.9* (±0.05)	1.2* (±0.2)	1.0* (±0.2)	0.5* (±0.1)

* Determined at 500 nm (see Materials and Methods).

By addition of sulfhydryl reagent at various times after illumination, further data are obtained. Addition of reagent 7 min after illumination at pH 6 resulted in mixed rates of reagent reaction consistent with the rates presented previously for the two dark and two light-exposed sulfhydryl groups (Tables II and III). However, addition of sulfhydryl reagent 8 h after illumination (thus permitting the complete thermal decay of metarhodopsin II_{380}) resulted in immediate reaction of three sulfhydryl groups ($t_{1/2}$ approx. 0.2 h) and a slowly reacting group corresponding in rate constant to the second dark SH group. The data indicate that the light-exposed sulfhydryl groups become freely available only after the thermal decay of metarhodopsin II_{380} and that the titration of the dark sulfhydryl groups before illumination had no observable effect on the thermal intermediates or sulfhydryl processes.

Data therefore show that the light-induced exposure of additional sulfhydryl groups of rhodopsin is associated with the thermal decay of metarhodopsin II_{380}.

Light-induced sulfhydryl exposure at high pH

At pH 8 three sulfhydryl groups become available after illumination and the rates of the sulfhydryl and intermediate reactions remain correlated but are faster than at lower pH (Table III). Spectrally one observes a decrease at 478 nm and an increase at 365 nm with an isosbestic at 410 nm (Fig. 4). It is not clear if metarhodopsin I_{478} is decaying directly to *N*-retinylidene-opsin$_{365}$ or through small amounts of metarhodopsin II_{380} (ref. 11) but the total process appears to involve the simultaneous exposure of three sulfhydryl groups. It seems to represent a more extensive disordering of the rhodopsin structure than at lower pH.

Reaction of the dark sulfhydryl groups

The reaction of the rhodopsin sulfhydryl groups in the dark appears to reflect the conformational state of the native rhodopsin and its sulfhydryl groups. Thus a rapidly reacting sulfhydryl and a slowly reacting sulfhydryl are observed (Table II). The rates of reaction suggest that the rapidly reacting group is located in a very accessible portion of the rhodospin while the other group is more protected. Some of the results are consistent with the idea that the rapidly reacting sulfhydryl group is exposed and susceptible to oxidation. As observed in Table II this group is often less than one sulfhydryl per rhodopsin. However, values of one sulfhydryl per rhodopsin may be obtained for this group when the rhodopsin solution is used within 1–2 h after preparation. The values approach those of Table II within 1 day. As a function of pH, the rapidly reacting group would appear to be more protected at the higher pH values. This is indicated by the slower reaction rates and better stoichiometry at the higher pH values (Table II).

The second dark sulfhydryl group is consistently a single group and shows no such pH dependence but does show certain rate changes as a function of pH. The slowest rate is observed at pH 7.0, suggesting that the group may be more buried at that pH and the molecule more compact.

Additional conclusions

The present results are consistent with previous work in this area (Table I) and appear to pinpoint the intermediate stage of exposure of the additional titratable sulfhydryl groups as the thermal decay of metarhodopsin II_{380}. Since that reaction is

quite slow even at physiological temperatures in intact retina[13], one cannot expect the sulfhydryl group exposure, or the conformation change that it represents, to be involved in any initial transduction processes.

It is of interest to note that though the additional sulfhydryl groups are exposed in the thermal decay of metarhodopsin II_{380}, that particular reaction has a negative entropy of activation[13], indicative of charge or conformational ordering. Recent experiments have shown that this reaction involves a proton release[11,15]. The proton release would result in a more negatively charged protein. It would therefore appear that the charge change of the rhodopsin may be a dominant factor in the entropy of activation while the sulfhydryl group exposure suggests that the molecule itself is either rearranging or disordering its structure.

Finally comparison of the present results with those obtained with silver Tris (Table I and ref. 3) indicate that two of the sulfhydryl groups available to silver Tris in the dark are not reactable with 4,4′-dithiodipyridine or 5,5′-dithio-bis-(2-nitrobenzoic acid). Because they are not available to the latter reagents, and because titration of those groups affected the viability of metarhodopsin I_{478} (ref. 3), it is reasonable to conclude that they are in a buried position in the rhodopsin molecule and may be close to or a part of the amino acids which are responsible for the metarhodopsin I_{478} conformation.

ACKNOWLEDGMENTS

This investigation was supported by Public Health Service Research Grant EY00413, Research Career Development Award (S.E.O., EY32951), and Cell Biology Training Grant (E.A.K., GM 01392).

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