Light-Dependent Phosphorylation of Rhodopsin: Number of Phosphorylation Sites†

Ursula Wilden and Hermann Kühn*

ABSTRACT: The light-dependent phosphorylation of bovine and frog rhodopsin was investigated under various conditions with suspensions of isolated rod outer segments. For both bovine rhodopsin and frog rhodopsin the average phosphorylation extent was found to be as high as 7.0 ± 0.3 mol of phosphate/mol of rhodopsin (7 P/R) under optimal incubation conditions. The same result was obtained with two independent analytical methods: with radiotracer analysis starting from [γ-^{32}P]ATP and with chemical phosphate analysis on affinity chromatographically purified phosphorylated rhodopsin. Phosphate incorporation in the dark was 50–100 times lower. Phosphorylated bovine rhodopsin was separated into rhodopsin fractions of different phosphorylation extents by means of ion-exchange chromatography, in order to get information about the distribution of *individual* phosphorylation extents on different rhodopsin molecules in a population of known *average* phosphorylation extent. The distribution was always found to be rather inhomogeneous. The highest individual phosphorylation extent found was 9 P/R; all intermediate phosphorylation extents between 0 and 9 P/R were also found. In highly phosphorylated preparations (average extent 7 P/R), about 35% of the rhodopsin contained 9 P/R, 22% contained 8 P/R, and the rest was a mixture of all phosphorylation extents between 0 and 7 P/R. During the time course of phosphorylation, lower phosphorylation extents predominated at earlier times and higher extents at later times of incubation, and some (2.5–3%) of the rhodopsin never became phosphorylated. This experiment suggests that there is some but not a strong cooperativity between the individual phosphorylation sites. High phosphate incorporation is favored under illumination conditions that allow photoregeneration and rebleaching of rhodopsin during the course of phosphorylation, but individual extents of 8 and 9 P/R are also obtained as a consequence of single-photon absorption. The time course of phosphorylation was found to be significantly accelerated by increasing the kinase vs. substrate ratio: either by producing less substrate, bleaching only part of the rhodopsin, or by adding soluble rhodopsin kinase.

Absorption of light by rhodopsin in rod outer segment (ROS)[1] membranes leads to a conformational change in the rhodopsin molecule (Kühn et al., 1982), making it available as a substrate for phosphorylation by a specific protein kinase and ATP (Kühn & Dreyer, 1972; Bownds et al., 1972; Kühn et al., 1973; Frank & Buzney, 1975; McDowell & Kühn, 1977). The terminal phosphate group from ATP, and also from GTP (Chader et al., 1980), is transferred to serine and threonine residues (Kühn & Dreyer, 1972; Hargrave et al., 1980) of bleached rhodopsin to form a stable phosphate ester. The kinase is independent of cyclic nucleotides (Kühn & Dreyer, 1972; Weller et al., 1975; Farber et al., 1979). The light-induced phosphorylation is followed by a slow dephosphorylation process, probably catalyzed by a phosphatase; about 1 h is required for complete dephosphorylation both in vivo (Kühn, 1974) and in vitro (Miller et al., 1977); this is the same time range that is also required for dark adaptation after strong illumination (Kühn et al., 1977).

There is general agreement that rhodopsin has more than one phosphorylation site. However, several significant points in this regard are still unclear and to some extent controversial; for instance, what is the number of phosphorylation *sites*, how many different *states* of phosphorylation exist at the rhodopsin molecule (just two states, namely, unphosphorylated and thoroughly phosphorylated, or also intermediate states), what is the function of the phosphorylated states, and are all or only certain of the rhodopsin molecules in ROS capable of undergoing light-induced phosphorylation?

The highest phosphorylation extents (P.-extents) reported so far have been 4 mol of phosphate *average*/mol of rhodopsin (4 P/R) in frog ROS at full bleaching (Miller et al., 1977) and 2.4 P/R in purified bovine ROS (Kühn & McDowell, 1977). So that information about *individual* P.-extents present in a population of phosphorylated ROS can be obtained, differently phosphorylated rhodopsin species must be separated from each other. Shichi et al. (1974), and more recently Shichi & Somers (1978), have separated phosphorylated from unphosphorylated bovine rhodopsin. They found that under their phosphorylation conditions, leading to an *average* incorporation of maximally 0.8 P/R, 84% of the total bleached rhodopsin remained unphosphorylated whereas the residual 16% was "highly" phosphorylated (5 P/R). They concluded that only rhodopsin located in certain regions of ROS was capable of being phosphorylated: preferentially rhodopsin in newly synthesized disks as suggested by double-labeling experiments. In contrast to this hypothesis, autoradiography of phosphorylated frog ROS by Paulsen & Schürhoff (1979) demonstrated a homogeneous distribution of the incorporated ^{32}P throughout the whole ROS.

We have reinvestigated the conditions necessary to obtain high phosphate incorporations. We find that under efficient conditions, more than 95% of the total rhodopsin in ROS can be phosphorylated. Average P.-extents of 7 P/R are reproducibly obtained; the maximum *individual* P.-extent is 9 P/R,

† From the Institut für Neurobiologie der Kernforschungsanlage Jülich, Postfach 1913, D-5170 Jülich, West Germany. *Received December 29, 1981.* This work was supported by SFB 160 from the Deutsche Forschungsgemeinschaft.

[1] Abbreviations: ROS, rod outer segments; P_i, inorganic phosphate; EDTA, ethylenediaminetetraacetic acid; P/R, moles of phosphate per mole of rhodopsin; P.-extent, phosphorylation extent; ECTEOLA–cellulose, epichlorohydrin triethanolamine–cellulose; ATP, adenosine 5′-triphosphate; Tris, tris(hydroxymethyl)aminomethane; GMP, guanosine 5′-monophosphate; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid.

0006-2960/82/0421-3014$01.25/0

and all intermediate individual P.-extents between 0 and 9 P/R are also found.

Materials and Methods

Materials. [γ-^{32}P]ATP (0.5–3 Ci/mmol) was purchased from Amersham Buchler and occasionally from New England Nuclear; it was stored in 50% aqueous ethanol at −70 °C with or without additions of ATP, NH_4HCO_3, and EDTA (see Results). 11-*cis*-Retinal was a kind gift from Dr. W. Sperling. Emulphogene BC-720 (General Aniline Co.) and Ammonyx LO (Onyx Chemical Co.) were kindly provided by Dr. J. H. McDowell and Dr. P. A. Hargrave.

Preparation of ROS. Bovine ROS were purified as described (McDowell & Kühn, 1977), with some modifications. All solutions contained 70 mM potassium phosphate, pH 7.0, 1 mM $MgCl_2$, 0.1 mM EDTA, and 1 mM dithiothreitol. About 100–200 freshly dissected bovine retinas were swirled with 100–150 mL of 45% (w/v) sucrose solution. Retinal debris were sedimented for 5 min at $3000g_{max}$ in a fixed-angle rotor. The supernatant, containing the ROS, was diluted with the same volume of buffer, underlayed with 0.77 M sucrose, and centrifuged as above. The crude ROS pellets were resuspended in 0.77 M sucrose with gentle shaking (no homogenization) and were further purified by centrifugation on stepwise sucrose gradients. The purified ROS, taken from the interface between 0.84 M sucrose and 1.00 M sucrose, were 1.5-fold diluted with buffer and sedimented for 20 min at $50000g_{max}$. The pellets were stored frozen at −70 °C under an argon atmosphere. The yield was 0.4–0.6 mg of rhodopsin/retina.

Frog ROS were shaken from dark-adapted frog (*Rana esculenta*) retinas in Ringer's solution. They were filtered through a nylon mesh and partially purified by sedimentation for 4 min at $1000g_{max}$. The ROS pellet was homogenized with buffer; the suspension was mildly sonicated and used directly for phosphorylation experiments. (Results presented in the figures and tables of this paper were obtained with *bovine* ROS except in Figure 3A where frog ROS were also used.)

Rhodopsin concentrations were determined in detergent solutions from the difference in A_{500} before and after bleaching in the presence of NH_2OH by assuming ϵ_{500} to be 41 000 and the molecular weight to be 38 000. All experiments with ROS membranes and with detergent-solubilized rhodopsin were performed in dim red light if not stated otherwise.

Phosphorylation Procedure. A frozen bovine ROS pellet was thawed and gently homogenized (glass/Teflon) in the appropriate buffer. Final concentrations were normally (unless stated otherwise in figure legends or in Table I) 0.5 mg of rhodopsin/mL, 100 mM sodium phosphate, pH 7.4, 3 mM [^{32}P]ATP (sp act. 10^3–10^4 cpm/nmol), and 1 mM $MgCl_2$. The suspension was transferred to a polycarbonate centrifuge tube and was, after addition of the [^{32}P]ATP, mildly sonicated for 1 min in a sonic cleaner bath (Buehler Ultramet III). It was then warmed to 30 °C for several minutes in a thermostated water bath. The reaction was started by the onset of illumination. Normally we used continuous illumination with white light from a 150-W reflector flood lamp, which bleached 80–90% of the rhodopsin within the first 20–30 s. The orange light occasionally used (Figure 6H) was as described by McDowell & Kühn (1977). In the experiment of Figure 7, white room light was used, which bleached 18% of the rhodopsin within 15 s. Incubation was always at 30 °C with bovine ROS and at 20 °C with frog ROS.

Aliquots of 100 μL were taken from the incubating mixture at distinct times and were pipetted into 300 μL of ice-cold 25% CCl_3COOH–20 mM ATP–5 mM H_3PO_4. The precipitated membranes were washed 3 times with 1-mL portions of 10% CCl_3COOH–5 mM H_3PO_4, by centrifugation in 1.5-mL Eppendorf vials. The final pellets were dissolved by shaking for 30–60 min in 1.5 mL of a toluene-based scintillation cocktail containing 10% (v/v) of the tissue solubilizer 0.6 N NCS (Amersham/Searle); the capped tubes were used directly for scintillation counting (Kühn & Wilden, 1982). Blank values (0.01–0.02 nmol of phosphate) were obtained from 100-μL aliquots of the incubation mixture taken before the onset of light. The ATP concentration was determined spectrophotometrically from stock solutions, and the specific radioactivity was determined from aliquots of the incubation mixture added directly to the NCS cocktail. In some experiments the incubation was carried out in individual Eppendorf vials, each containing 100 μL of incubation mixture, rather than in one large vial, leading to the same results.

Phosphate incorporations are given in moles of phosphate per mole of rhodopsin (P/R). Virtually all of the ^{32}P incorporated into the membranes is in fact bound to rhodopsin, as has been shown previously by gel electrophoresis (Kühn & Dreyer, 1972) and will be confirmed by the column chromatographic procedures of this report.

Regeneration with 11-cis-Retinal. Since [^{32}P]opsin is unstable in detergents, it must be regenerated to [^{32}P]rhodopsin prior to solubilization in detergents. A 3–4-fold molar excess of 11-*cis*-retinal in ethanol was added to the incubation mixture in the dark after phosphorylation. Regeneration was allowed to take place for 3 h at 30 °C; the mixture was mildly sonicated (see above) from time to time. If less than 97% of the rhodopsin was regenerated, an equimolar portion of 11-*cis*-retinal was added and the mixture was further incubated at 20 °C overnight. The final regeneration yield was 100 ± 2%.

The membranes were then washed 4–5 times by centrifugation (20 min at 40000*g*) with 100 mM phosphate until the supernatant was free of ^{32}P. One last washing was performed with water. The phosphorylation extent was determined again at the end of the washing procedure.

Affinity Chromatography on Concanavalin A–Sepharose. Phosphorylated, regenerated, and washed ^{32}P-labeled ROS membranes were extracted with detergent (2% Emulphogene or 2% Ammonyx LO) containing 20 mM NaCl in 1 mM sodium phosphate, pH 7.4. The extract was put on a column of concanavalin A–Sepharose 4B (Pharmacia) equilibrated with the same detergent solution. The column bed volume was 0.5–10 mL, depending on the amount of rhodopsin (binding capacity about 2 mg of rhodopsin/mL of bed volume). The column was washed with about 10 times its bed volume of the above detergent solution and then with two additional bed volumes of 1 mM sodium phosphate–2% detergent until the effluent was free of 280 nm absorbing material. Rhodopsin was then eluted with 2% detergent containing 200 mM methyl α-D-mannoside (Sigma, grade III) and 1 mM sodium phosphate, at a very slow flow rate (taking at least 15 min for the total elution from the smallest columns in order to allow for equilibration).

Ion-Exchange Chromatography on ECTEOLA–Cellulose ("Column Cascade"). A number (3–20) of little columns arranged on top of each other were filled with epichlorohydrin triethanolamine–cellulose (ECTEOLA–cellulose; Bio-Rad), thoroughly washed with 2% Emulphogene in 1 mM sodium phosphate, pH 6.0. (Ocassionally, Ammonyx LO was used, but the binding capacity of the columns for [^{32}P]rhodopsin was higher with Emulphogene.) Since the binding capacity of ECTEOLA–cellulose is much greater for highly than for low

phosphorylated rhodopsins, the first columns of the cascade were always smaller than late columns. The columns were run by their hydrostatic pressure.

The [^{32}P]rhodopsin sample, prepurified on concanavalin A–Sepharose in 2% Emulphogene–1 mM sodium phosphate, was applied to the first (uppermost) column and allowed to flow serially through all of the columns. Each column bound as much [^{32}P]rhodopsin as was allowed by its binding capacity, and the excess [^{32}P]rhodopsin therefore went on to the next column. Unphosphorylated rhodopsin did not bind to any of the columns and was recovered in the final effluent from the last column. The column cascade was washed with 2% Emulphogene–1 mM sodium phosphate until the effluent from the last column was free of rhodopsin. The [^{32}P]rhodopsin bound to each column was then eluted in one step with 2% Emulphogene–300 mM NaCl–100 mM sodium phosphate, pH 7.0. Highly phosphorylated rhodopsins were found in the effluents of early columns and lower phosphorylated rhodopsins in later columns. This procedure avoids exposure of highly phosphorylated rhodopsins to excess ECTEOLA–cellulose and therefore minimizes denaturation of [^{32}P]rhodopsin; such denaturation (loss of A_{500}) was always observed if one large column, eluted with a salt gradient (Shichi & Somers, 1978), was used rather than the column cascade. Amounts and phosphorylation extents of [^{32}P]rhodopsin in the fractions were determined by measuring A_{500} and ^{32}P. If the ratio of A_{280}/A_{500} was to be determined, the fractions had to be purified again on small concanavalin A–Sepharose columns in order to remove some UV-absorbing material that was always eluted from ECTEOLA–cellulose at high salt concentrations. This ratio was between 1.65 and 1.75 for all fractions tested (measured in Emulphogene solution).

Phosphate Determination. The phosphate bound to [^{32}P]rhodopsin, after its purification and delipidation on concanavalin A–Sepharose in 2% Ammonyx LO, was determined by using the method of Ames (1966). Five nanomoles of [^{32}P]rhodopsin was applied to a single assay. Each assay was repeated at least 4 times. Blank values (obtained from assays in the absence of rhodopsin) were subtracted. All assays were performed in quartz tubes to lower the blank level. The assay was calibrated by a series of 36 samples (10–60 nmol of P_i) of standard phosphate buffer.

Results

Optimum Incubation Conditions for High Phosphorylation Extents. The following conditions were found to reproducibly yield average phosphate incorporations as high as 7 mol of phosphate/mol of rhodopsin (7 P/R): 3 mM [^{32}P]ATP, 1 mM $MgCl_2$, and 100 mM sodium or potassium phosphate buffer (or 100 mM Tris-HCl), pH 7.4; ROS at 0.5 mg of rhodopsin/mL; continuous illumination with white light. This P.-extent of 7 P/R represents an average value measured for the whole population of bleached rhodopsin molecules; analysis of the P.-extents of individual rhodopsin molecules within this population will be presented later in this paper (Figures 4–7). First we shall discuss some of the parameters that influence the rate and maximum extent of phosphate incorporation.

Concentration of [^{32}P]ATP and $MgCl_2$. Millimolar concentrations of [^{32}P]ATP are required to get high final extents of phosphate incorporation (Figure 1A). At 0.1 mM ATP, a final extent of only 0.8–1 P/R is reached, in agreement with other reports [e.g., Shichi & Somers (1978)], although the molar excess of ATP over rhodopsin was 8-fold in this sample (Figure 1A, lowest curve). The ATP concentration in intact frog ROS has been reported to be about 3 mM (Robinson & Hagins, 1979).

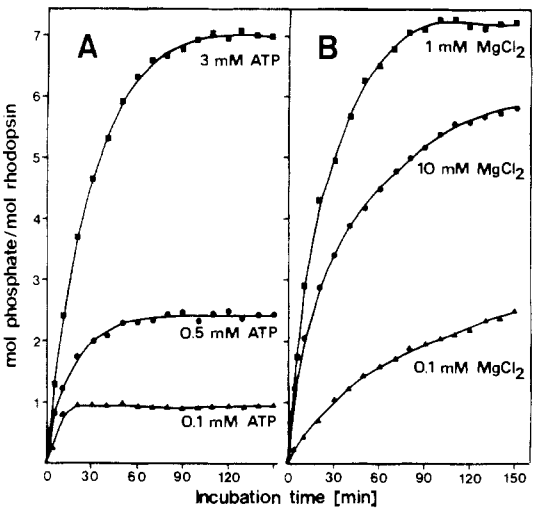

FIGURE 1: Influence of concentration of ATP and $MgCl_2$ on the rate and final level of rhodopsin phosphorylation. Continuous illumination in 100 mM sodium phosphate buffer, pH 7.4. The ordinate is the same for (A) and (B). (A) Constant (1 mM) $MgCl_2$ concentration at various ATP concentrations: (■) 3; (●) 0.5; (▲) 0.1 mM ATP. (B) Constant (3 mM) ATP concentration at various $MgCl_2$ concentrations: (■) 1; (●) 10; (▲) 0.1 mM $MgCl_2$.

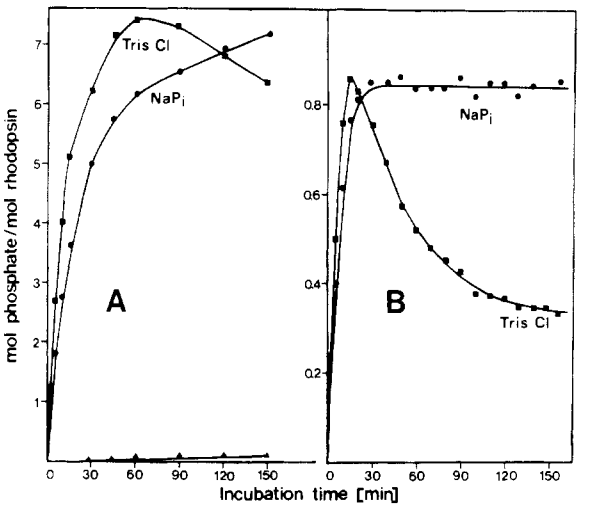

FIGURE 2: Influence of inorganic phosphate on phosphorylation and dephosphorylation of rhodopsin at various ATP concentrations. Continuous illumination with white light at pH 7.4 in all cases except filled triangles (dark incubation in 100 mM sodium phosphate). (A) Optimum incubation conditions: 3 mM [^{32}P]ATP, 1 mM $MgCl_2$, and 100 mM sodium phosphate (●) or 100 mM Tris-HCl (■), respectively. (B) 0.1 mM [^{32}P]ATP, 4 mM $MgCl_2$, and 60 mM sodium phosphate (●) or 60 mM Tris-HCl (■), respectively.

Magnesium ion is required; EDTA inhibits (Kühn & Dreyer, 1972), but excess $MgCl_2$ is also inhibitory (Figure 1B). The highest incorporations were obtained when the concentration of $MgCl_2$ was about 30–50% of the [^{32}P]ATP concentration. The inhibitory effect of excess $MgCl_2$ was observed in Tris-HCl buffer (not shown), as well as in phosphate buffer, and was also observed under different conditions by Chader et al. (1980).

Concentration and Type of Buffer. Since rhodopsin kinase is not stable at low ionic strength (Kühn, 1978), the salt concentration should be above 50 mM. The type of buffer ions used may influence the initial rate of phosphorylation but has little influence on the maximum phosphorylation extent (Figure 2). For instance, the initial rate is somewhat faster in 100 mM Tris-HCl than in 100 mM sodium phosphate buffer at the same pH, both at high (Figure 2A) and at low (Figure 2B) ATP concentrations. Furthermore, phosphate

Table I: Effect of Monovalent Cations on Initial Rate and Maximum Extent of Rhodopsin Phosphorylation

incubn conditions (concn in mM)				initial rate[a]	max extent	
Tris-HCl	[^{32}P]ATP	$MgCl_2$	other additions	(P/R per 5 min)	[$(P/R)_{max}$]	no. of expt
150	3	1		2.0 ± 0.5	7.0 ± 0.2	4
50	3	1	100 NaCl	1.4 ± 0.3	7.0 ± 0.2	4
50	3	1	100 KCl	1.3 ± 0.2	7.0 ± 0.3	4
160	0.1	4		0.57 ± 0.05	1.05 ± 0.19	5
60	0.1	4	100 NaCl	0.52 ± 0.02	0.90 ± 0.10	5
60	0.1	4	100 KCl	0.57 ± 0.04	1.09 ± 0.11	5
	3	1	100 sodium phosphate	1.8 ± 0.5	7.0 ± 0.3	10
	3	1	100 potassium phosphate	1.7 ± 0.5	7.0 ± 0.3	3

[a] The initial rate was determined by using the incorporation at 5 min of incubation.

buffer completely blocks dephosphorylation of phosphorylated rhodopsin. In Tris buffer, dephosphorylation is observed at low ATP (Figure 2B) and even to some extent at high ATP concentration (Figure 2A), even in continuous illumination. Therefore we normally used phosphate buffer for the preparation of ^{32}P-labeled ROS with high and stable phosphorylation extents.

The rate and maximum extent of phosphorylation is only little influenced by the presence of Na^+ or K^+ ions under a variety of different conditions (Table I). This is in contrast to observations by Shichi & Somers (1978), who reported a strong and specific inhibition by Na^+ ions (90% inhibition by 100 mM NaCl). The reason for this discrepancy is not clear.

Influence of the Preparation. Final phosphorylation extents of 7 P/R have been obtained reproducibly with a large number (20) of ROS preparations over a period of several years. However, the initial rate of phosphate incorporation varied somewhat among different ROS preparations (Figure 3A), probably due to different amounts of soluble rhodopsin kinase (Kühn, 1978) present in the different preparations. Accordingly, addition of kinase-containing extract to an ROS suspension significantly increased the phosphorylation rate (Figure 3B). The dark incorporation into unbleached ROS was normally 0.1–0.2 P/R after 2 h of incubation (see Figure 2A). The maximum light-induced P.-extent of frog ROS was similar to that of bovine ROS but was reached more rapidly, in spite of the lower temperature used for frog ROS (Figure 3A).

Illumination Conditions. The highest phosphorylation extents (7 P/R) are obtained with continuous strong white light. Continuous orange light ($\lambda < 540$ nm) leads to somewhat lower extents (on the average 6 P/R). The higher efficiency of white light is most probably due to "recycling" (photoreisomerization) of the chromophore, which leads to a continuous turnover of photoregenerated and rebleached rhodopsin (McDowell & Kühn, 1977). Since the capacity of rhodopsin to be phosphorylated decays with time after bleaching (Kühn, 1978), a continuous supply of freshly bleached rhodopsin produced by recycling obviously increases the probability of multisite phosphorylation at long incubation times. The rate of recycling is strongly reduced in orange light (McDowell & Kühn, 1977).

Determination of Phosphorylation Extents by P_i Analysis. During the first 1–2 years of this investigation we observed routinely that apparent P.-extents of 7 P/R could only be obtained by using freshly delivered [^{32}P]ATP (but not with all batches of [^{32}P]ATP). Already within the first week after delivery, the apparent P.-extents of rhodopsin, determined from the ^{32}P incorporation, decreased significantly; typical decreases were about 20% per month (see Table II, third column). Possible explanations for this decrease were as follows: (i) an inhibitor of rhodopsin kinase might be formed as a product

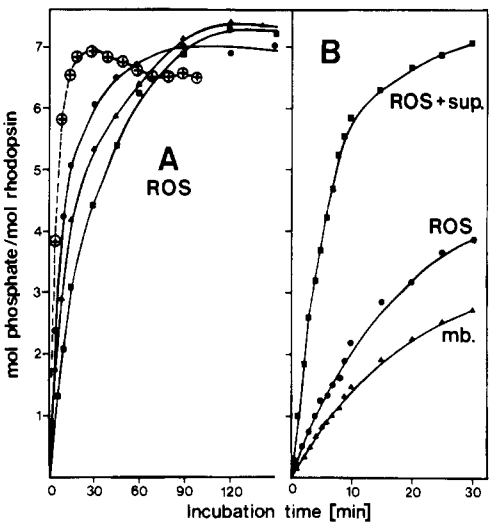

FIGURE 3: Time course of rhodopsin phosphorylation in various preparations. The incubation medium was 100 mM sodium phosphate, pH 7.4, 3 mM [^{32}P]ATP, and 1 mM $MgCl_2$ at 30 °C for bovine ROS (solid lines) and 10 mM Hepes, pH 7.2, 115 mM NaCl, 3 mM [^{32}P]ATP, and 1 mM $MgCl_2$ at 20 °C for frog ROS (dashed line). The ordinate is the same for (A) and (B). (A) Three different bovine ROS preparations symbolized by the different filled symbols and a frog ROS preparation (open circles with crosses). (B) Dependence of the phosphorylation rate on the amount of soluble kinase present. [Middle curve (ROS)] A relatively slow phosphorylating bovine ROS preparation without additions. An aliquot (12 mg of rhodopsin) of this preparation was extracted for soluble kinase with 20 mM Tris-HCl–2 mM EDTA; the supernatant was made 100 mM in Tris-HCl, pH 7.4, and 3 mM in $MgCl_2$ and was added to another part of the same ROS [2 mg of rhodopsin; upper curve (ROS + sup.)]. The lower curve (mb.) shows phosphorylation of the extracted and resuspended membranes. Rhodopsin concentration in all incubations was 0.5 mg/mL.

of radiochemical decomposition of the [^{32}P]ATP, leading to really lower P.-extents; (ii) no inhibitor was formed but the radiochemical decomposition was in fact as high as 20% per month; this would lead to reproducibly high "real" P.-extents but to too low "apparent" P.-extents because the measured specific radioactivity would include other ^{32}P-labeled products besides [^{32}P]ATP.

Chemical analysis of P_i bound to [^{32}P]rhodopsin after its regeneration and purification showed that the second explanation (ii) was correct (Table II). The real P.-extents were constantly 7 P/R, and the apparent P.-extents obtained with aged [^{32}P]ATP were wrong due to considerable ^{32}P-labeled byproducts. Subsequently we were able to better protect the [^{32}P]ATP against radiochemical decomposition by adding cold ATP (to 60–120 mM), EDTA (to 1 mM), and NH_4HCO_3 (to 10 mM) shortly after delivery. Some [^{32}P]ATP batches, however, delivered from two different manufacturers, gave

Table II: Inorganic Phosphate Analysis as a Control To Determine Phosphate Incorporation Independently of [^{32}P]ATP[a]

preparation	age of [^{32}P]ATP	incorpd mol of phosphate/mol of rhodopsin: apparent (from ^{32}P)	incorpd mol of phosphate/mol of rhodopsin: determined by P_i anal.
ROS			66 ± 4
purified rhodopsin			0.0 ± 0.5
purified [^{32}P]rhodopsin	"fresh"	7.3	7.1 ± 0.4
purified [^{32}P]rhodopsin	5 weeks	5.6	6.9 ± 0.3
purified [^{32}P]rhodopsin	10 weeks	4.3	7.2 ± 0.5

[a] Rhodopsin was purified on concanavalin A–Sepharose, resulting in total removal of phospholipids (compare first and second row). Frozen aliquots of the same ROS preparation were phosphorylated for 150 min under optimum conditions (see beginning of Results), with the same batch of [^{32}P]ATP, at different times after delivery of the [^{32}P]ATP. The [^{32}P]ATP was stored in 50% ethanol at −70 °C without additions. Apparent P.-extents were determined as usual from the specific radioactivity of the [^{32}P]ATP (always 5000 cpm/nmol). Inorganic phosphate was determined after regeneration of the [^{32}P]opsin with 11-*cis*-retinal and purification on concanavalin A–Sepharose. 5 nmol of rhodopsin was applied to each assay (except for the case of ROS, 0.5–1 nmol of rhodopsin). Data are given ± standard deviation for eight individual determinations.

lower apparent P.-extents (5–6 P/R) from the beginning on, regardless of the high "radiochemical purity" indicated by the manufacturers; in these cases again P_i analysis revealed real P.-extents of 7 P/R.

Table II also shows that the purification on concanavalin A–Sepharose columns leads to complete separation of rhodopsin from the phospholipids present in ROS. We found that more than 95% of the ^{32}P from the column effluents was associated with the purified rhodopsin fraction and that no detectable ^{32}P was present in the lipid-containing fractions. The apparent P.-extent of rhodopsin was the same before and after chromatography. This clearly demonstrates that in these ^{32}P-labeled ROS preparations virtually all of the membrane-bound ^{32}P is bound to rhodopsin and none is associated with phospholipid. In contrast, Shichi & Somers (1978) proposed that a major fraction of ^{32}P in their ^{32}P-labeled ROS was associated with phospholipids (without giving evidence for the phospholipid nature of these fractions).

Separation of Differently Phosphorylated Rhodopsins. The phosphorylation extents described so far in this report are *average* extents with no information about the P.-extents of single rhodopsin molecules in the mixture. We wanted to check if in our highly phosphorylated ROS preparations of average 7 P/R, all of the rhodopsin was phosphorylated to the same extent or if individual P.-extents other than 7 P/R could be found. Using the separation technique on ECTEOLA–cellulose columns as described by Shichi & Somers (1978), we obtained some separation but were not able to do satisfactory quantitative analysis of P.-extents since the [^{32}P]-rhodopsin turned out to be unstable on these columns. Up to 50% of the A_{500} material (rhodopsin) applied to the columns got lost during chromatography whereas over 95% of the ^{32}P was recovered, leading to an apparent increase in P.-extents. This denaturation was partly due to pH changes occurring during the salt-gradient elution.

These difficulties were avoided by using an alternative chromatographic procedure (the column cascade; see Materials and Methods) in which the [^{32}P]rhodopsins, particularly the highest phosphorylated fractions, are exposed to minimum amounts of ECTEOLA–cellulose for relatively short times. In principle, a number of small columns arranged serially was used rather than one big column. After saturation of the binding capacity of the first column with highly phosphorylated rhodopsin, the effluent containing the nonbound rhodopsin flowed through the second column and so on. Each column was thus loaded with the maximum amount of [^{32}P]rhodopsin allowed by its binding capacity. Elution of bound [^{32}P]-rhodopsin from the columns was performed by a single step of high salt and buffer concentration whereby relatively high rhodopsin concentrations were obtained and the losses were minimal.

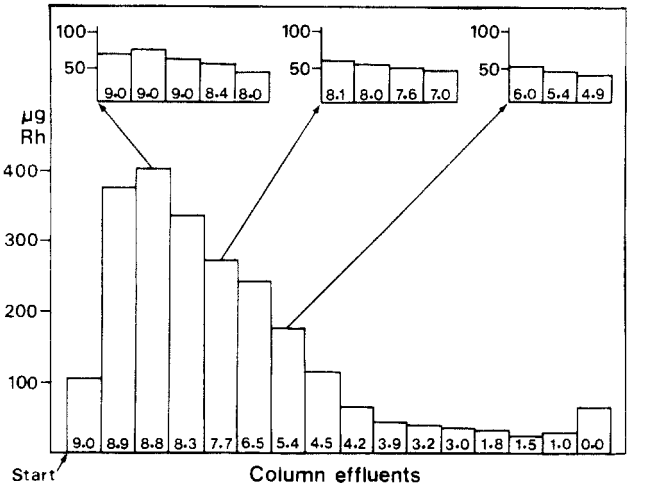

FIGURE 4: Separation of differently phosphorylated rhodopsins by anion-exchange chromatography. A highly phosphorylated ^{32}P-labeled ROS preparation (average P.-extent 7.0 P/R; 2.5 mg of rhodopsin) was regenerated, solubilized in Emulphogene, purified on a concanavalin A–Sepharose column, and then applied to a cascade of 15 small columns of ECTEOLA–cellulose arranged serially from left to right. Each bar in the figure represents the effluent of one column (for details see Materials and Methods). The amount of rhodopsin (indicated by the height of the bars on the ordinate) present in each fraction depends on the binding capacity (size) of the corresponding column. Column sizes ranged from 0.2 (first column) to 4 mL (last four columns). Phosphorylation extents (in P/R) of the effluents are written at the bottom of each bar. Highly phosphorylated rhodopsins have the highest affinity to the anion exchanger and are therefore bound to and eluted from early columns (left side) and lower phosphorylated rhodopsins from later columns (right side). The last bar to the right (unphosphorylated rhodopsin) represents the fraction that ran through all the columns without being bound. (Insets) Some of the fractions, as indicated by the arrows, were rechromatographed each on a second little cascade of three to five columns; this demonstrates that each fraction from the first column is a mixture of essentially two adjacent P.-extents.

The separation of a highly phosphorylated (average 7 P/R) preparation is shown in Figure 4. Obviously the individual rhodopsin molecules are not homogeneously phosphorylated: all P.-extents between 0 and 9 P/R are found. The individual P.-extent of 9 P/R is surprisingly high. Several lines of evidence demonstrated that it is real and not an artifact due to possible denaturation of rhodopsin (loss of A_{500}) on the columns. (i) The recovery of both A_{500} and ^{32}P from the column cascade was always 90–95%. (ii) The average P.-extent never changed during chromatography: recalculation of an average P.-extent from the sum of the fractions lead to the same extent as in the original sample before chromatography. (iii) The A_{280}/A_{500} ratio even of the highly phosphorylated fractions (8–9 P/R) was always between 1.65 and 1.75, indicating negligible contamination with [^{32}P]opsin (see Materials and Methods). Higher P.-extents than 9.0 P/R were never found,

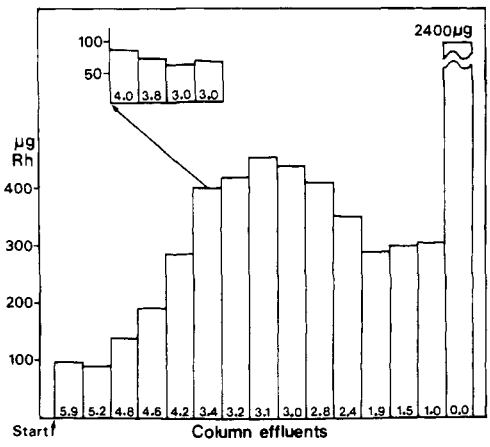

FIGURE 5: Separation of a ^{32}P-labeled ROS preparation with relatively low phosphorylation extent (average 2 P/R) on a cascade of 14 ECTEOLA–cellulose columns. Details are as in Figure 4.

not even when the first columns of the cascade were kept very small in order to become saturated by the binding of only the most extensively phosphorylated rhodopsin species.

The question arises whether the intermediate P.-extents between 1 and 8 P/R measured in fractions of the column cascade (Figure 4) really represent P.-extents of 1–8 P/R present at individual rhodopsin molecules or just various mixtures of unphosphorylated and fully phosphorylated (9 P/R) rhodopsin, due to incomplete separation. We investigated this by mixing highly phosphorylated fractions (9 P/R) with unphosphorylated rhodopsin in detergent. The mixtures were desalted by using a concanavalin A–Sepharose column and were then analyzed on an ECTEOLA–cellulose column cascade. The column cascade yielded only fractions with 0 and 9 P/R in several such mixing experiments, indicating complete separation of the two components and no formation of mixed fractions.

Some of the fractions from a first column cascade were desalted on concanavalin A–Sepharose, and each was rechromatographed on a second ECTEOLA–cellulose column cascade (Figure 4, insets). The results indicate that each fraction from the first cascade consists of a mixture of essentially two adjacent P.-extents. In the second cascade only fractions with these two adjacent P.-extents, and again mixtures of them, were found. This provides further evidence that all of the P.-extents between 1 and 9 really exist and can reproducibly be separated from each other.

A typical separation of ^{32}P-labeled ROS of relatively low average P.-extent (2 P/R) is shown in Figure 5. In this case, all P.-extents between 0 and 6 P/R were found in the mixture. About one-third of the rhodopsin remained unphosphorylated. Rechromatography of one of the fractions again yielded only the two adjacent P.-extents.

Distribution of Individual Phosphorylation Extents during the Time Course of Phosphate Incorporation. Since most of the fractions of the ECTEOLA–cellulose column cascade consist of essentially two adjacent P.-extents, one can estimate the amounts of the two "pure" P.-extents present in each fraction. For example, a fraction of 5.34 P/R is estimated to contain 66% of rhodopsin with 5 P/R and 34% with 6 P/R. The relative error margin of this evaluation is estimated to be about ±15%; it is due to the possible presence of small amounts of other than the two adjacent P.-extents in fractions, but it will at least partially be averaged out for the total evaluation. By adding up the amounts of each individual P.-extent found in the various fractions and plotting its sum vs. the corresponding P.-extent, we obtained the type of "histogram" shown in Figure 6.

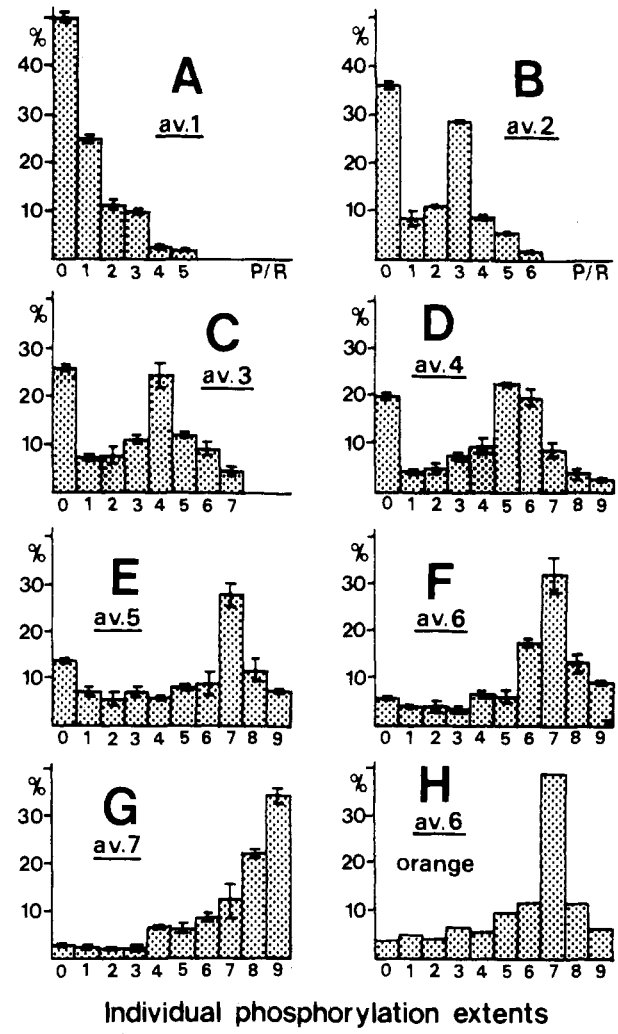

FIGURE 6: Time course of the distribution of individual phosphorylation extents. In samples A–G, aliquots of the same ROS suspension were subjected to phosphorylation in continuous bright white light for various time periods, leading to various average P.-extents as indicated by the number on each diagram: (A) 3 min, average 1 P/R; (B) 5 min, 2 P/R; (C) 10 min, 3 P/R; (D) 16 min, 4 P/R; (E) 25 min, 5 P/R; (F) 43 min, 6 P/R; (G) 90 min, 7 P/R. Incubation times that lead to whole-numbered average P.-extents were determined in preliminary experiments with an aliquot of the same ROS preparation. Phosphorylation reactions were terminated by the addition of 20 mM EDTA. Sample H was incubated for 150 min in orange light ($\lambda >$ 540 nm). Each sample was regenerated, detergent-solubilized, purified on concanavalin A–Sepharose, and then separated on an ECTEOLA–cellulose column cascade as described in the legend to Figure 4 and under Materials and Methods. The relative amounts of rhodopsin at individual P.-extents were calculated from the column fractions as described in the text and were plotted (in percent) on the ordinates. The abscissas represent individual P.-extents (moles of phosphate per mole of rhodopsin). The whole experiment was performed twice with two different ROS preparations; the individual results are indicated by the small "error bars" around the average values given by the large bars.

Figure 6A–G shows the distribution of individual P.-extents in seven samples of differing average P.-extents, obtained by incubating an ROS suspension with [^{32}P]ATP–$MgCl_2$ for various periods of time. It is obvious that each sample, obtained at short as well as at long incubation times, represents a mixture of different individual P.-extents. At early times of incubation, the lower individual P.-extents predominate; at later times (25–45 min), the 7 P/R species predominates, and only in extensively phosphorylated preparations (90 min) the highest P.-extents of 8 and 9 P/R predominate. Significant

amounts of 9 P/R are first observed after about 16 min of incubation (average 4 P/R). The amount of residual unphosphorylated rhodopsin decreases continuously from 50% (1 min; average 1 P/R) to 2–3% (90 min; average 7 P/R). The results were rather similar for the two ROS preparations tested in Figure 6.

Analysis of the time course of phosphorylation as shown in Figure 6 indicates that the mechanism of sequential phosphorylation of the different sites at each rhodopsin molecule must be rather complex. Phosphorylation of one site obviously does not facilitate phosphorylation of the other sites to a large extent, since in this case only unphosphorylated and highly phosphorylated rhodopsins should be found. On the other hand, some facilitation seems to take place, indicated by the presence of relatively high amounts of unphosphorylated rhodopsin particularly at early times (low average P.-extents). The data of Figure 6A–G do not indicate that particular P.-extents are especially favored (except perhaps the extent of 7 P/R); it appears that the affinities of the different phosphorylation states to further bind kinase and undergo further phosphorylation are not very different from each other. In any event, the data suggest that the kinase must act several distinct times on the same rhodopsin molecule in order to highly phosphorylate it.

Influence of Illumination Conditions on Distribution of Individual Phosphorylation Extents. Most of the phosphorylation experiments of this report were performed in continuous bright white light, which favors "recycling" of bleached rhodopsin by photoreisomerization and rebleaching (see above) during the course of phosphate incorporation. The question arises whether high individual P.-extents can only be obtained under such conditions or also under more gentle illumination conditions. Figure 6H shows an analysis of a ^{32}P-labeled ROS sample that was phosphorylated under continuous *orange* light illumination in which all of the rhodopsin is bleached but the rate of recycling is strongly reduced (McDowell & Kühn, 1977). The average P.-extent did not exceed 6 P/R. Rhodopsins with individual P.-extent of 7 P/R predominated in the mixture (Figure 6H); however, rhodopsins with 8 and 9 P/R were also found in significant amounts.

So that the possibility of recycling during phosphorylation could be completely eliminated, some ROS preparations were partially bleached (13–23%) by a short-term light exposure (10–20 s) in the absence of ATP, and phosphorylation was then allowed to proceed in the dark after addition of [^{32}P]ATP. Figure 7 shows an experiment in which 18% of the rhodopsin was bleached. The final level of phosphate incorporation (4.6 P/R bleached) was reached much faster than in a fully bleached control sample (Figure 7A), probably because of a higher kinase vs. substrate ratio in the partially bleached sample (see also Figure 3B). In this case, the predominant P.-extent in the mixture was 6 P/R, but even here P.-extents of 8 and 9 P/R were also present (Figure 7B). About 4% of the bleached rhodopsin contained 9 P/R. This clearly demonstrates that absorption of a single photon is sufficient for the incorporation of nine phosphate groups into the bleached rhodopsin molecule. Recycling obviously facilitates (Figure 6G) but is not required for the 9-fold phosphorylation of single rhodopsin molecules.

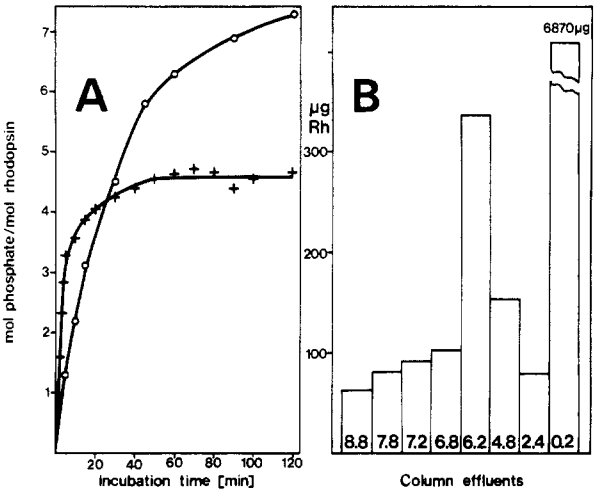

FIGURE 7: Phosphorylation of rhodopsin at partial bleaching extent. (A) Time course of phosphate incorporation in 100 mM sodium phosphate, 3 mM [^{32}P]ATP, and 1 mM $MgCl_2$, pH 7.4: (+) dark incorporation into ROS in which 18% of the rhodopsin had been bleached by a 15-s light exposure prior to addition of [^{32}P]ATP; (○) phosphate incorporation into the same ROS preparation under continuous illumination with bright white light is shown for comparison. The ordinate shows moles of phosphate incorporated per mole of *bleached* rhodopsin. (B) The partially bleached (18% R*) ^{32}P-labeled ROS preparation was regenerated, detergent-solubilized, and analyzed on a cascade of ECTEOLA–cellulose columns for its distribution of individual P.-extents, as described in the legend of Figure 4. This shows that up to nine phosphates can be incorporated in the dark into previously bleached rhodopsin.

Discussion

The experiments demonstrate that rhodopsin contains nine sites for light-induced phosphate incorporation. This is an extraordinarily high number of phosphorylation sites for a polypeptide chain not greater than 40 000 daltons. Multisite phosphorylation has been reported for a number of other proteins [see also reviews by Cohen (1976) and Krebs & Beavo (1979); the highest phosphorylation extent of an enzyme we could find in the literature was six phosphates bound per 96 000-dalton subunit of glycogen synthase (Smith et al., 1971)]. We established the number of nine phosphorylation sites per rhodopsin by two independent methods: ^{32}P radiotracer analysis and direct phosphate analysis.

Hargrave et al. (1980) have shown that seven potential phosphorylation sites (three serine and four threonine residues) are located close to each other, near the carboxyl terminus of rhodopsin. This region comprised about 85% of the total ^{32}P of their [^{32}P]rhodopsin preparations. The residual two (?) phosphorylation sites are not located in the hydrophilic carboxyl-terminal region that comprises about 40 amino acids (P. A. Hargrave, personal communication) and must therefore be located farther away in the polypeptide chain, separated from the major phosphorylation domain by at least two hydrophobic transmembrane α helices [see Hargrave et al. (1980), rhodopsin model]. It thus appears that rhodopsin has at least two distinct phosphorylatable domains separated by large sections of the polypeptide chain. It is unknown whether the two domains are topographically close to each other on the cytoplasmic-exposed surface of rhodopsin.

Under our optimum incubation conditions we obtained an *average* P.-extent of 7 P/R reproducibly for 20 different ROS preparations. The light-induced phosphate incorporation was 50–100 times greater than the dark incorporation, indicating a high specificity of the kinase reaction. In these highly phosphorylated preparations, about 35% of the total rhodopsin contained nine phosphates bound (see Figures 4 and 6). The reason why all of the rhodopsin molecules do not become phosphorylated to this high extent is unknown. It may be due to inhomogeneities in the arrangement (availability) of rhodopsin at various positions in the disk or plasma membrane or to the different age of different disks (Young, 1967). About 2–3% of the total rhodopsin always remained un-

phosphorylated. This amount corresponds approximately to the relative amount of plasma membrane surface in ROS, but there is no obvious reason why the plasma membrane bound rhodopsin should not become phosphorylated.

In any event, our results show clearly that at least 95% of the rhodopsin in ROS membranes can be phosphorylated, in contrast to reports (Shichi et al., 1974; Shichi & Somers, 1978) suggesting that most rhodopsin molecules cannot be phosphorylated and that the rhodopsin in freshly synthesized, proximal disks is preferentially phosphorylated. Shichi & Somers (1978) used inhibitory phosphorylation conditions (high $MgCl_2$, low [^{32}P]ATP) that lead to average P.-extents not higher than 0.8 P/R; this explains why most of the rhodopsin remained unphosphorylated. Under these conditions we found similarly low average P.-extents (see Figure 2B).

The results from the ECTEOLA–cellulose column cascades indicate that not only unphosphorylated and extensively (9 P/R) phosphorylated rhodopsins exist but also all intermediate P.-extents between 1 and 8 P/R are present in medium and highly phosphorylated preparations. This is not an artifact due to incomplete separation of unphosphorylated and extensively phosphorylated rhodopsin, since rechromatography of fractions, and of mixtures of highly phosphorylated and unphosphorylated fractions, yields consistent and reproducible separations (see Results). The same type of distribution of P.-extents was found in a nonionic detergent (Emulphogene, Figures 4–7) and in a zwitterionic detergent (Ammonyx LO, results not shown). Furthermore, earlier separation experiments, using isoelectric focusing in various detergents including octyl glucoside, have also indicated multiplicity of phosphorylation states of rhodopsin (Kühn & McDowell, 1977; H. Kühn, unpublished results).

Therefore, one is faced with the complex situation that rhodopsin can exist in at least 10 different states of phosphorylation (0–9 P/R). The situation may be even more complex since it is not known if a "pure" phosphorylation extent is really homogeneous with respect to the site(s) phosphorylated; for example, whether in a purified preparation of 1.0 P/R all of the phosphate is bound to the same serine or threonine residue or distributed among different residues.

The distribution of individual P.-extents seems to depend on the illumination conditions. Several conditions have been tested: short-term (Figure 5) and long-term (Figure 4) continuous illumination with bright white light (see also Figure 6A–G); long-term continuous illumination with moderate orange light (Figure 6H); and phosphorylation in the dark after a partial bleaching with moderate white light (Figure 7). The formation of highly phosphorylated rhodopsins is obviously favored when the conditions allow recycling of rhodopsin by multiple-photon absorption (continuous white light). However, P.-extents of 8 and 9 P/R are also obtained upon single-photon absorption in the absence of recycling (Figure 7). This raises the possibility that such high P.-extents also occur in physiological conditions.

The physiological function of the phosphorylation reaction is not yet determined. It has been proposed to function as a "turn-off" mechanism for the light-induced production (Kühn et al., 1973) or degradation (Liebman & Pugh, 1980) of a putative internal transmitter (Ca^{2+} or cyclic GMP, respectively). The multiplicity of phosphorylated forms of rhodopsin demonstrated in this study suggests, however, that its function is probably more complex than just a simple turn-on/turn-off effect. Since phosphorylation of rhodopsin is not an "all-or-none" but a graded phenomenon, it seems more likely that it may regulate some process in the rod cell in a *graded* way. It is also possible that differently phosphorylated rhodopsins may have quite different functions. Perhaps the functional heterogeneity is restricted to a few different phosphorylation *domains*.

In any event, any hypothesis about a function of rhodopsin phosphorylation should from now on specify which phosphorylated species is concerned, and experiments designed to test such hypotheses should preferentially be performed with purified preparations of homogeneously phosphorylated rhodopsin. Highly phosphorylated rhodopsin will certainly exhibit a large electrostatic effect on its environment by having seven to nine negative charges highly locally concentrated; this may influence the overall electric properties of the membrane, the binding of certain ions, and/or the interaction with peripheral enzyme proteins.

The time course of phosphorylation of the different sites appears to be rather complex (Figure 6). It suggests a certain but not a large extent of cooperativity between the sites. The overall time course of phosphorylation that appears to be slow in these high-bleach experiments is certainly faster under more physiological conditions of high membrane (and protein) concentration and low light intensities. Bleaching large amounts of rhodopsin within a short time obviously produces an unusually large amount of substrate for the kinase such that the amount of kinase becomes rate limiting. Consistent with this assumption is the observation that the overall phosphorylation rate is considerably accelerated both by adding soluble kinase to fully bleached ROS (Figure 3B) and by bleaching only part of the total rhodopsin (Figure 7); both treatments lead to a higher kinase vs. substrate ratio. This leaves open the possibility that the light-induced phosphorylation of rhodopsin in vivo may in fact be a rather rapid reaction.

Acknowledgments

We thank O. Mommertz for her expert technical assistance, Dr. W. Sperling for the generous gift of 11-*cis*-retinal, and Drs. P. A. Hargrave and J. H. McDowell for critical reading of the manuscript.

References

Ames, B. N. (1966) *Methods Enzymol. 8*, 115–118.
Bownds, D., Dawes, J., Miller, J., & Stahlman, M. (1972) *Nature (London), New Biol. 237*, 125–127.
Chader, G. J., Fletcher, R. T., Russell, P., & Krishna, G. (1980) *Biochemistry 19*, 2634–2638.
Cohen, P. (1976) *Trends Biochem. Sci. (Pers. Ed.) 1*, 38–40.
Farber, D. B., Brown, B. M., & Lolley, R. N. (1979) *Biochemistry 18*, 370–378.
Frank, R. N., & Buzney, S. M. (1975) *Biochemistry 14*, 5110–5117.
Hargrave, P. A., Fong, S.-L., McDowell, J. H., Mas, M. T., Curtis, D. C., Wang, J. K., Jusczak, E., & Smith, D. P. (1980) *Neurochem. Int. 1*, 231–244.
Krebs, E. G., & Beavo, J. A. (1979) *Annu. Rev. Biochem. 48*, 923–959.
Kühn, H. (1974) *Nature (London) 250*, 588–590.
Kühn, H. (1978) *Biochemistry 17*, 4389–4395.
Kühn, H., & Dreyer, W. J. (1972) *FEBS Lett. 20*, 1–6.
Kühn, H., & McDowell, J. H. (1977) *Biophys. Struct. Mech. 3*, 199–203.
Kühn, H., & Wilden, U. (1982) *Methods Enzymol. 81*, 489–496.
Kühn, H., Cook, J. H., & Dreyer, W. J. (1973) *Biochemistry 12*, 2495–2502.

Kühn, H., McDowell, J. H., Leser, K.-H., & Bader, S. (1977) *Biophys. Struct. Mech. 3*, 175–180.
Kühn, H., Mommertz, O., & Hargrave, P. A. (1982) *Biochim. Biophys. Acta 679*, 95–100.
Liebman, P. A., & Pugh, E. N. (1980) *Nature (London) 287*, 734–736.
McDowell, J. H., & Kühn, H. (1977) *Biochemistry 16*, 4054–4060.
Miller, J. A., Paulsen, R., & Bownds, M. D. (1977) *Biochemistry 16*, 2633–2639.
Paulsen, R., & Schürhoff, K. (1979) *Eur. J. Cell Biol. 19*, 35–39.
Robinson, W. E., & Hagins, W. A. (1979) *Nature (London) 280*, 398–400.
Shichi, H., & Somers, R. L. (1978) *J. Biol. Chem. 253*, 7040–7046.
Shichi, H., Somers, R. L., & O'Brien, P. J. (1974) *Biochem. Biophys. Res. Commun. 61*, 217–221.
Smith, C. H., Brown, N. E., & Larner, J. (1971) *Biochim. Biophys. Acta 242*, 81–88.
Weller, M., Virmaux, N., & Mandel, P. (1975) *Proc. Natl. Acad. Sci. U.S.A. 72*, 381–385.
Young, R. W. (1967) *J. Cell Biol. 33*, 61–72.

Energetics of the Equilibrium between Two Nucleotide-Free Myosin Subfragment 1 States Using Fluorine-19 Nuclear Magnetic Resonance†

John W. Shriver and Brian D. Sykes*

ABSTRACT: A new fluorine-containing reagent has been synthesized and used to specifically label the reactive sulfhydryl [sulfhydryl-1 (SH_1)] of myosin subfragment 1 (S-1). The labeled S-1 (S-1-CF_3) demonstrates activated calcium and magnesium adenosinetriphosphatase (ATPase) activities relative to S-1 and a lower potassium ethylenediaminetetraacetate (EDTA) ATPase activity. Maximal effect is obtained with the modification of one thiol per S-1. The ^{19}F NMR spectrum of S-1-CF_3 contains only one resonance with a line width of 110 Hz, which implies a rotational correlation time of 2.3×10^{-7} s. The chemical shift of this resonance is sensitive to temperature, pH, ionic strength, and nucleotides bound in the active site. The temperature dependence of the chemical shift clearly indicates two limiting states for the S-1-CF_3 with a highly temperature-dependent equilibrium between 5 and 40 °C. The low-temperature state appears to be identical with the state resulting from the binding of Mg·ADP or Mg·AMPPNP at 25 °C. The energetics of the conformational change have been studied under various conditions. At pH 7 in 25 mM cacodylate, 0.1 M KCl, and 1 mM EDTA, $\Delta H°$ = 30 kcal/mol and $\Delta S°$ = 105 cal deg^{-1} mol^{-1}. A decrease in pH to 6.5 results in an increased population of the low-temperature state with $\Delta H°$ = 31 kcal/mol and $\Delta S°$ = 107 cal deg^{-1} mol^{-1}. Similarly, the low-temperature state is favored by low ionic strength. In 5.8 mM piperazine-*N*,*N*′-bis(2-ethanesulfonic acid) and 1 mM EDTA (pH 7), $\Delta H°$ = 8 kcal/mol and $\Delta S°$ = 27 cal deg^{-1} mol^{-1}. We have also obtained ^{19}F NMR spectra of S-1-CF_3 in D_2O solution with 30% ethylene glycol at pH 7.1. Increasing concentrations of ethylene glycol progressively stabilize the high-temperature state.

Recent work with ^{31}P nuclear magnetic resonance (NMR)[1] (Shriver & Sykes, 1980, 1981a,b) and ultraviolet and fluorescence spectroscopy (Morita, 1977; Bechet et al., 1979) has indicated that myosin subfragment 1 can exist in two discrete states or conformations in the presence of ADP or AMPPNP. Transient kinetic experiments have provided evidence for two myosin S-1 ADP and AMPPNP complexes in addition to a transitory recognition complex (Trybus & Taylor, 1979). In addition, transient and steady-state kinetic experiments have provided evidence for two M·ADP·P ternary complexes (Bagshaw & Trentham, 1974). This body of work has implied that there are two fundamental states of myosin subfragment 1 with the relative population of the two states being dependent on the nucleotide occupying the ATPase active site and the temperature. However, to this date, no evidence for two states of S-1 in the absence of nucleotide has been provided. We show here that a fluorine probe attached to the SH_1 of myosin S-1 may be used as a sensitive monitor of the conformational state of S-1 in the absence of nucleotide. The variation with temperature of the chemical shift of this probe clearly demonstrates that myosin S-1 in the absence of nucleotide may exist in two discrete states which differ significantly in structure as indicated by large $\Delta H°$ and $\Delta S°$ values for the transition. As seen with the two M·ADP and M·AMPPNP states, $\Delta G° \approx 0$ at physiological temperatures.

A wide variety of probes have been attached to the SH_1 sulfhydryl of myosin S-1 (Quinlivan et al., 1969; Seidel et al., 1970; Takashi et al., 1976; Nihei et al., 1974; Thomas, 1978), and the effects of the modification on the characteristics of the ATPase have been well documented. Aromatic derivatives of iodoacetamide have been shown to have a high specificity

† From the Medical Research Council Group on Protein Structure and Function, Department of Biochemistry, University of Alberta, Edmonton, Alberta, Canada T6G 2H7. *Received October 20, 1981.* This work has been supported by the Medical Research Council of Canada (postdoctoral fellowship to J.W.S. and grant to the Group on Protein Structure and Function) and by the Muscular Dystrophy Association of Canada (postdoctoral fellowship to J.W.S.).

[1] Abbreviations: SH_1, sulhydryl-1; S-1, myosin subfragment 1; S-1-CF_3, myosin subfragment 1 labeled with *N*-[4-(trifluoromethyl)-phenyl]iodoacetamide; DTT, dithiothreitol; ADP, adenosine 5′-diphosphate; AMPPNP, adenosine 5′-(β,γ-imidotriphosphate); ATPase, adenosinetriphosphatase; EDTA, ethylenediaminetetraacetic acid; Pipes, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid); Tris, 2-amino-2-(hydroxymethyl)-1,3-propanediol; Mes, 2-(*N*-morpholino)ethanesulfonic acid; NMR, nuclear magnetic resonance.

0006-2960/82/0421-3022$01.25/0