Nearest Neighbor Relationships of the Polypeptides in Ubiquinone Cytochrome *c* Reductase (Complex III)†

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ABSTRACT: Ubiquinone cytochrome *c* reductase (complex III) in detergent dispersion has been cross-linked with two reversible cross-linking agents dithiobissuccinimidylpropionate and dimethyl-3,3′-dithiobispropionimidate and the cross-linked products formed have been analyzed by two-dimensional gel electrophoresis. Under mild reaction conditions, polypeptides I and II, II and VI, I and V, and VI and VII were the most prominent subunit pairs seen. With higher levels of reagent, larger aggregates were produced until an aggregate of apparent molecular weight 310 000 was the dominant band on gels. This is the complex III monomer.

Ubiquinone cytochrome *c* reductase or complex III is an oligomeric protein which probably spans the mitochondrial inner membrane and is involved in electron transport and in the coupling of this reaction to ATP synthesis or ion transport. The complex contains b heme, c_1 heme, and nonheme iron centers in the molar ratio 2:1:1 (Rieske, 1976). Eight different polypeptides have been identified as components of complex III (Bell and Capaldi, 1976). Each has been purified in a denatured form and the identities of some polypeptides have been determined as follows: polypeptide I, core protein I (mol wt 50 000); II, core protein II (46 000); III, cytochrome *b* (31 500); IV, cytochrome c_1 (29 000); V, nonheme iron protein (25 000); VI, a polypeptide which copurifies with the cytochrome c_1 hemoprotein (14 000); VII, a polypeptide which cofractionates with cytochrome *b* (12 500); VIII, antimycin binding protein (9000) (Bell and Capaldi, 1976).

The arrangement of components in complex III is not known. As a first step in determining the structure of this complex, we have used two cleavable cross-linking agents, dithiobissuccinimidylpropionate (DSP)[1] and dimethyl 3,3′-dithiobispropionimidate dihydrochloride (DTBP), to establish some of the nearest neighbor interactions among the component polypeptides. Both reagents gave a number of cross-linked products including polypeptide pairs and larger aggregates.

Experimental Section

Complex III was prepared by the method of Rieske (1967). Small aliquots of the enzyme (20–50 mg/mL) were stored at −20 °C. Protein concentrations were determined by the method of Lowry et al. (1951). Heme *b* and c_1 concentrations were estimated as described by Williams (1964). Ubiquinone cytochrome *c* reductase activity was measured basically as described by Leung and Hinkle (1975) but without addition of exogenous phospholipid and using a CoQ_2 analogue with a ten-carbon side chain as substrate (kindly provided by Dr. Bernard Trumpower, Dartmouth Medical School).

Enzyme was prepared for cross-linking by diluting to 1 or 2 mg/mL in 0.05 M triethanolamine hydrochloride buffer (pH 8.0) containing 0.1% Triton X-100, 0.5% Triton X-100, or 0.33% deoxycholate. The protein solution was dialyzed against several changes of buffer to remove residual ammonium sulfate which would react with the cross-linker. Immediately before use, DTBP was dissolved at 20 mg/mL in the triethanolamine hydrochloride buffer. DSP was dissolved at the same concentration in Me_2SO. The cross-linking reaction was started by adding the bifunctional reagent to give a final concentration between 0.05 and 2.0 mg/mL. Reaction times were varied between 5 and 30 min. All experiments were conducted at room temperature. The reaction was stopped by quenching with 50 μL of 1 M ammonium acetate per mL of reaction mixture. Samples were concentrated to 15–20 mg/mL with dry sucrose and dissociated in 3% sodium dodecyl sulfate by heating to 60 °C for 5 min or to 100 °C for 1 min before being applied to 7.5 or 10% polyacrylamide gels prepared by the method of Weber and Osborn (1969). Samples of highly cross-linked enzyme were run on 4% Biophore gels using the buffer system 0.205 M Tris, 0.205 M acetic acid, and 0.1% sodium dodecyl sulfate, pH 6.4.

Two-dimensional gel electrophoresis was performed essentially as described by Briggs and Capaldi (1977) using the Swank–Munkres gel system (Swank and Munkres, 1971) for the second dimension. The slab gel contained 10% acrylamide monomer and 0.67% bisacrylamide. Gels were fixed and stained as described by Downer et al. (1976). Densitometric traces of the gels were made at 550 nm with a Gilford linear transport attachment to a Beckman DU spectrometer. Gels were calibrated for molecular weight determination using thyroglobulin, β-galactosidase, γ-globulin, glyceraldehyde-3-phosphate dehydrogenase, carbonic anhydrase, cytochrome *c*, cross-linked bovine serum albumin, and cross-linked aldolase. The known molecular weights of the polypeptides of complex III were also used in constructing standard curves.

Results

Resolution of Cross-Linked Products. Complex III obtained by the method of Rieske (1967) contained 7.1–8.0 nmol of *b* heme and 3.5–4.0 nmol of c_1 heme per mg of protein and had a molecular activity of 15–20 μmol of cytochrome *c* reduced min^{-1} (mg of protein)$^{-1}$ (under the reaction conditions described in the Experimental Section). The complex was cross-linked with DTBP or DSP at various concentrations of

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[1] Abbreviations used: DSP, dithiobissuccinimidylpropionate; DTBP, dimethyl-3′-dithiobispropionimidate dihydrochloride; mol wt, molecular weight; Tris, tris(hydroxymethyl)aminomethane.

TABLE I: Subunit Pairs and Other Aggregates Resolved after Cross-Linking with the Cleavable Bifunctional Reagents.

Mol wt of product	Subunits involved	Sum of mol wt of subunits	Cross-linker	Conditions	
				mg/mg	Time (min)
310 000	I–VIII	250 000[c]	DSP	1	30[b]
130 000	I, II, V	121 000	DSP	0.1	5
130 000			DTBP	0.5	15
115 000	I, II, VI	110 000	DSP	0.1	5
115 000			DTBP	0.5	15
105 000	I, II	96 000	DSP	0.1	5
105 000			DTBP	0.5	15
86 000	I, V	75 000	DSP	0.1	5
82 000			DTBP	0.5	15
67 000	II, VI	60 000	DSP	0.1	5
62 000			DTBP	0.5	15
73 000	VII, I, or II	57 500[a]	DSP	0.5	15
29 000	VI, VII	26 500[a]	DSP	0.1	5
32 000			DTBP	0.5	15

[a] The anomalous migration of VII in the first dimension makes it difficult to predict the expected molecular weight. [b] Product identified on 4% gels. Other products listed were resolved on 7.5% gels. [c] Calculated from c_1 heme content of the complex.

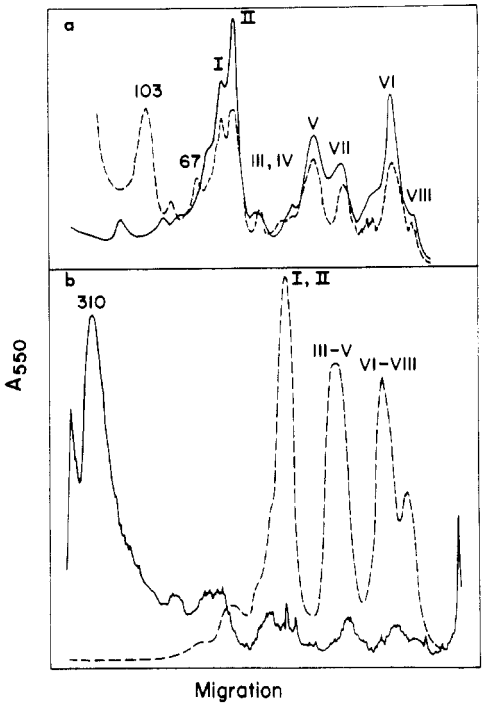

FIGURE 1: Densitometric traces of sodium dodecyl sulfate gels showing the effects of cross-linking complex III. The upper traces show a 7.5% sodium dodecyl sulfate gel run in the Weber-Osborn buffer system (but without β-mercaptoethanol) of complex III cross-linked with DSP at 0.1 mg/mg protein for 5 min. The dashed line shows the cross-linked sample and the solid line is the control. The lower traces show a 4% gel run in the Tris-acetate buffer system of complex III cross-linked with DSP at 1.0 mg/mg for 30 min. The solid line is the cross-linked sample, the dashed line untreated enzyme.

reagent and for different reaction times and the disappearance of individual subunits and appearance of cross-linked products were analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis. Experiments were performed on enzyme dissolved in either Triton X-100 or deoxycholate.

The effect of DSP on the polypeptide profile of complex III is shown in Figure 1. With low levels of reagent (0.1 mg/mg protein; incubated for 5 min) several cross-linked products were formed with molecular weights below 120 000 (Figure 1a). Under these reaction conditions 90% of the ubiquinone cytochrome *c* reductase activity was retained. With higher levels of cross-linker, larger aggregates were stabilized. At 1.0 mg of DSP/mg of protein (30 min), the enzyme was cross-linked into an aggregate of molecular weight 310 000 which dominated the gel scan. Under these reaction conditions individual polypeptides virtually disappeared from the gel profile (Fig. 1b). The fully cross-linked complex retained 5% of the initial electron transfer activity.

DTBP was also effective in cross-linking complex III, but at higher concentrations of reagent. With 0.5 mg of DTBP per mg of complex III (incubated for 15 min), the major cross-linked products formed had molecular weights below 120 000 (Figure 3 and Table I). Under these conditions, the enzyme retained 75% of its initial ubiquinone cytochrome *c* reductase activity. Higher levels of DTBP generated larger aggregates with a concomitant loss of activity.

Identification of the Subunits Contributing to the Various Cross-Linked Aggregates. The two bifunctional reagents used in this study could be cleaved by sulfhydryl reagents to regenerate the component polypeptides of any cross-linked product. For optimal resolution, two-dimensional gel electrophoresis was used and cross-linked aggregates were separated in the horizontal dimension and each aggregate was analyzed for the subunits it contained in the vertical dimension. In different experiments 4, 7.5, or 10% cylindrical gels run in either the Tris-acetate buffer system (for 4% gels) or in the Weber-Osborn gel system were used. Protein was electrophoresed out of this gel through an agarose layer containing β-mercaptoethanol and into a 10% gel in the Swank-Munkres gel system which acted as the vertical dimension. A control of unreacted complex III run in this two-dimensional system is shown in Figure 2. The cylindrical gel contained around 400 μg of protein which is the amount needed to resolve some of the cross-linked products formed. Bands were identified from their migration in the vertical dimension. In some experiments unreacted complex III was run along one edge of the gel to act as a marker for each polypeptide. In Figure 2, the major polypeptides of complex III are labeled I–VIII based on the order in which they run on the Swank-Munkres gel. Several impurities could be seen on the overloaded gel. For example, the component labeled SD_1 is the flavin containing polypeptide of succinate dehydrogenase (mol wt 73 000). The smaller

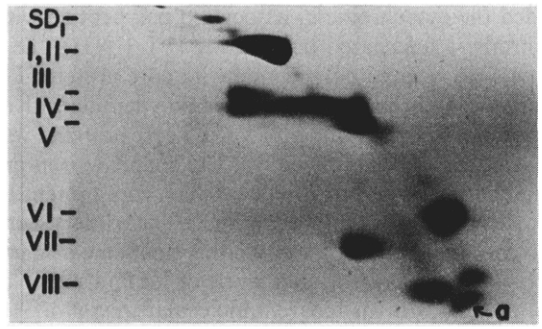

FIGURE 2: Two-dimensional electrophoresis of complex III using a 10% Weber-Osborn gel in the first dimension and a Swank-Munkres gel system in the vertical dimension.

subunit of succinate dehydrogenase is probably present too but it runs close enough to polypeptide V that it would not be seen as a discrete spot on this gel. The component labeled a is seen commonly in complex III preparations. It has not been ruled out that this polypeptide is an integral part of complex III.

Assuming that polypeptides migrate on gels as a function of their molecular weight, they should run on a diagonal across the slab in two-dimensional gel electrophoresis. However, some of the polypeptides of complex III do not behave ideally but migrate differently under different conditions of gel electrophoresis. In the buffer systems used here polypeptides I, II, V, VI, and VIII ran close to a diagonal but polypeptide IV (cytochrome c_1), polypeptide III (cytochrome b), and polypeptide VII (the cytochrome b associated component) all ran off and below the diagonal. The anomalous behavior of IV resulted from not having β-mercaptoethanol in the dissociating medium for the first dimension. When complex III was dissociated in sodium dodecyl sulfate and reducing agent prior to electrophoresis, polypeptide IV ran on or very close to the diagonal. The anomalous migration of cytochrome c_1 in gels in the absence of reducing agents has also been noted by Gellerfors and Nelson (1975).

Polypeptides III and VII in contrast ran off the diagonal whether a reducing agent was used in dissociating the complex or not, although the broadness of band III in the first dimension was greater when β-mercaptoethanol was omitted.[2] In most experiments, polypeptide III was distributed in a molecular weight range from 50 000 to 30 000 in the horizontal dimension (Weber-Osborn gel) but was focused in the second dimension (Swank-Munkres gel) between 32 000 and 28 000 daltons. The likely explanation of this banding pattern is that cytochrome b is not fully unfolded in sodium dodecyl sulfate alone but is denatured completely by the combination of sodium dodecyl sulfate and 8 M urea in the Swank-Munkres gel (see Yu et al., 1975; Capaldi et al., 1977).

Polypeptide VII also ran with a higher molecular weight in the horizontal dimension than in the vertical direction. The position of this component in the horizontal dimension depended on the percentage acrylamide used in making the gel as well as on the length of the gels used. On 10% gels (10 cm length), polypeptide VII ran ahead of subunit V and with a molecular weight of 26 000. There is no obvious explanation for this anomalous behavior.

An analysis of the cross-linked products obtained by reacting complex III with low levels of DTBP is shown in Figure 3. The

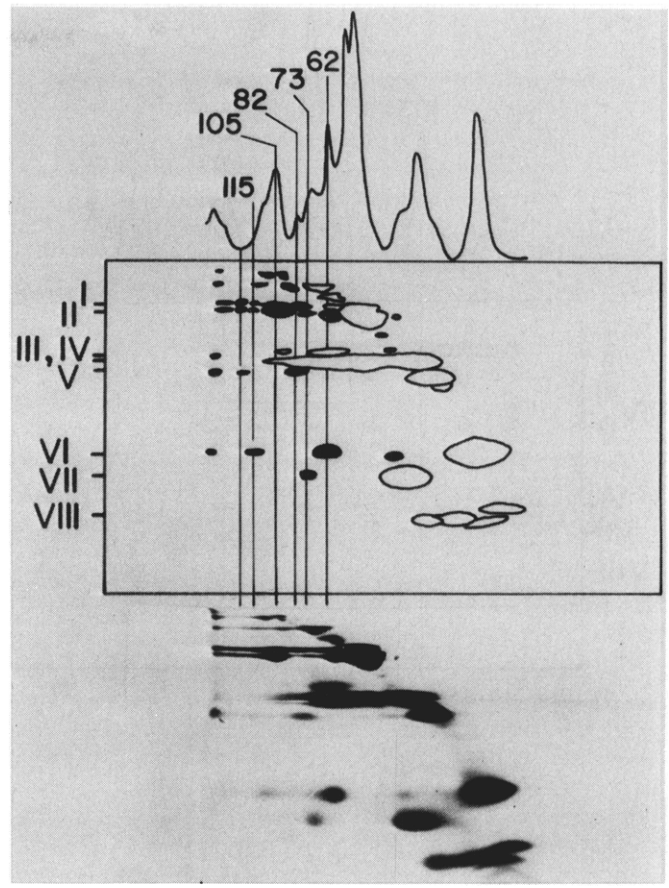

FIGURE 3: Two-dimensional analysis of the cross-linked aggregates generated with DTBP (0.5 mg/mg for 15 min). The gel trace shows the cross-linked products formed, identified by their apparent mol wt on the gel. The slab gel in the lower part of the figure shows the spots generated by cleaving the cross-linked products with β-mercaptoethanol and these are drawn schematically in the center of the figure. Blank spots represent spots seen on the control; black spots are off diagonal spots derived from cross-linked products.

polypeptide profile of cross-linked enzyme is shown in the upper part of the figure. The schematic representation of the two-dimensional gel in the lower part of the figure identifies off-diagonal spots which were not present in the control. The most prominent cross-linked product (mol wt 105 000) was found to be a dimer of polypeptides I and II. Other dimers were resolved in this experiment including II plus VI, and I plus V. An aggregate comigrating with the succinate dehydrogenase flavoprotein (mol wt 73 000) was also seen containing polypeptides VII and either I or more likely II. Two trimers, I, II plus V and I, II plus VI, were also identified. Other spots were seen off the diagonal that were not present in the control but their partner or partners were not obvious.

An analysis of cross-linked aggregates obtained by reacting the enzyme complex with DSP is shown in Figure 4. Again the most prominent cross-linked product was the I plus II dimer with II plus VI and VI plus VII also being resolved in this gel. In other experiments using similar conditions, an aggregate containing I and V was also seen. Highly cross-linked complex III was also analyzed on two-dimensional gels. The major band generated by high levels of DSP (Figure 1, gel b) was found to contain all of the polypeptides of the complex (results not shown). The experiments shown in Figures 3 and 4 were performed on enzyme dissolved in Triton X-100. The same results were obtained on enzyme dissolved in deoxycholate.

The many cross-linked products seen with DTBP and DSP

[2] The smearing of band III can be mostly avoided by dissociating samples for electrophoresis in sodium dodecyl sulfate and high concentrations of β-mercaptoethanol by heating to 100 °C but these conditions would of course cleave the cross-linked aggregates.

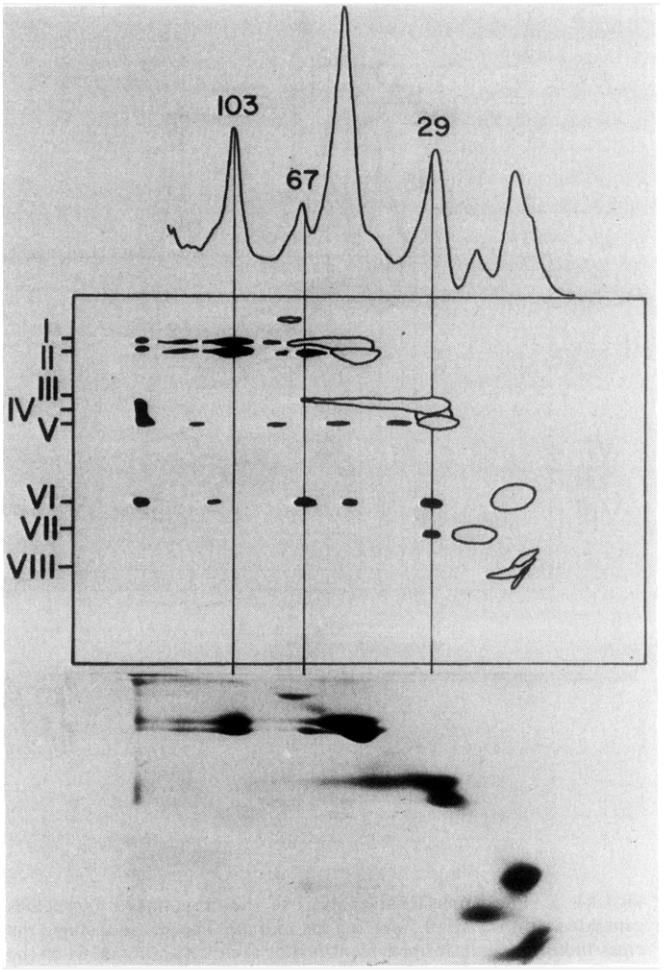

FIGURE 4: Two-dimensional analysis of the cross-linked aggregates obtained with DSP (0.1 mg/mg for 5 min). The gel trace shows the cross-linked products found. A schematic and the original slab gel show the components in the various aggregates generated.

in both Triton X-100 and deoxycholate are summarized in Table I. The molecular weight of aggregates determined from the gels is in most cases higher than that calculated by summing the molecular weights of component polypeptides.

Discussion

Chemical cross-linking of neighboring polypeptides is a useful approach to determining the arrangement of components in an oligomeric complex (Davies and Stark, 1970; Lutter et al., 1974; Thomas and Kornberg, 1975; Wang and Richards, 1974; Briggs and Capaldi, 1977). Analysis of the components in cross-linked products has been facilitated by the introduction of reversible cross-linking agents such as DSP and DTBP which are cleaved in the presence of sulfhydryl reagents to regenerate the components of any aggregate (Wang and Richards, 1974; Bragg and Hou, 1975). Interacting components can then be identified directly on gels rather than being assessed from the molecular weight of the aggregate.

In this study DSP and DTBP have been used to identify the nearest neighbor relationships in ubiquinone cytochrome *c* reductase. At low levels of either reagent, several dimers, and trimers containing different polypeptides were resolved. This places the components in these aggregates within 11 Å of each other in the complex. Among the cross-linked products resolved a dimer of core protein I and II was present in the largest amount as judged by staining intensity. Other dimers resolved included the cytochrome c_1 associated polypeptide with the cytochrome *b* associated polypeptide (VI + VII), the cytochrome c_1 associated polypeptide with core protein II (II + VI), and the cytochrome *b* associated polypeptide with either core proteins I or II. A dimer of VI and core protein I was not seen. Another dimer resolved was the nonheme iron protein (V) and core protein I. A dimer between core protein II and the nonheme iron protein was not seen. The trimers seen contained core protein I and II with either nonheme iron protein or polypeptide VI. With high levels of DSP, a major cross-linked product with an apparent molecular weight of 310 000 was generated which contained all of the different polypeptides in complex III. There was very little cross-linked material of higher molecular weight in either deoxycholate or Triton X-100 solubilized enzyme. The minimum molecular weight of complex III calculated from the cytochrome c_1 concentration is 250 000 (Hatefi et al., 1962; Rieske, 1976). The large aggregate produced by cross-linking is therefore a monomer rather than a dimer of complex III and the smaller cross-linked products (i.e., dimers, trimers of different polypeptides) must be formed by intracomplex cross-linking.

In summary, cross-linking with DTBP or DSP has identified the near neighbors for several of the polypeptides in complex III. Only polypeptides III, IV, and VIII were not cross-linked into dimers or trimers. With III and IV the problem is that these polypeptides smear out across the two-dimensional gel used in resolving the cross-linked products, making it difficult to identify either component in smaller molecular weight aggregates. The anomalous behavior of both resulted from using less than optimal conditions for dissociating complex III for electrophoresis. To resolve cytochrome *b* (III) and cytochrome c_1 (IV) on gels, it is necessary to dissociate by warming in sodium dodecyl sulfate in the presence of β-mercaptoethanol but these are conditions which would cleave DSP or DTBP. Recently, reversible cross-linking agents with a tartaryl group joining the functional moieties have been synthesized (Lutter et al., 1974; Coggins et al., 1976). With such reagents, cross-linked products can be separated after samples have been dissociated in the presence of β-mercaptoethanol, without disrupting the cross-linker which is only cleaved by periodate treatment. Experiments with such reagents are underway and these may identify interactions between cytochrome *b*, cytochrome c_1, and other components of complex III.

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Control of Light-Activated Phosphorylation in Frog Photoreceptor Membranes†

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ABSTRACT: In this paper, we examine some factors which regulate the efficiency of light in activating rhodopsin phosphorylation. We have measured phosphate incorporation after illumination in suspensions of bullfrog rod outer segments incubated with [γ-^{32}P]ATP. We observed that delaying ATP addition after illumination causes maximum phosphate incorporation to decrease 80% within 2 h. This decay occurs in urea-treated, extracted rod outer segment membranes. The decay of the light effect is not influenced by regeneration of opsin to rhodopsin or the presence of long-lived photoproducts. However, regeneration of opsin increases the amount of phosphorylation initiated by a second exposure to light. Further phosphorylation can also occur after phosphate groups have been removed from the membranes by dephosphorylation. Finally, we have confirmed our earlier observation that small amounts of light (bleaching less than 5% of the rhodopsin present) are more effective, by tenfold, in initiating phosphorylation than are larger amounts.

In rod outer segments from vertebrate retinas, light triggers a sequence of reactions that convert the pigment rhodopsin to intermediate photoproducts and finally to opsin and all-*trans*-retinal (Hubbard et al., 1965). Alongside that classical photoproduct sequence, other light-dependent reactions have recently been discovered. Cyclic nucleotide levels in the rod outer segments seem to be regulated by light-dependent reactions (Keirns et al., 1975). In addition, illumination of rhodopsin triggers a Mg^{2+}-dependent transfer of the terminal phosphate group from ATP or GTP to serine and threonine residues on the protein moiety opsin. This light-activated phosphorylation of photoreceptor membranes has been observed in rod outer segments of frog (Bownds et al., 1972; Kühn, 1974) and cattle (Kühn and Dreyer, 1972; Frank et al., 1973; Shichi et al., 1974; Weller et al., 1975c; Chader et al., 1975). Inhibitors of the phosphorylation affect the light-induced permeability change of the outer segment plasma membrane, measured in vitro. In the presence of these inhibitors, small amounts of light produce greater changes in sodium permeability (Miller et al., 1975). Therefore, the phosphorylation reactions may be part of the mechanism linking rhodopsin photochemistry to the outer segment receptor potential and its control.

In this paper, we examine three factors which may control the effectiveness of light in activating phosphorylation: (1) dark reactions taking place in the receptor membrane after illumination, (2) the number of phosphate groups already bound to the rod outer segments, and (3) the proportion of the visual pigment that has been converted by light to photoproducts.

Materials and Methods

Rod outer segments were shaken into Ringer's solution from retinas of bullfrogs, which had been dark adapted for 12 h. The Ringer's solution contained 115 mM NaCl, 2 mM KCl, 2 mM $MgCl_2$, and 10 mM Hepes (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid), pH 7.5. The outer segments were sedimented by centrifugation at 1200*g* for 2 min at 22 °C and then resuspended in Ringer's solution. Portions of this suspension were illuminated with orange light calibrated to bleach known amounts of rhodopsin (Paulsen et al., 1975) and [γ-^{32}P]ATP of known specific activity was added to a final concentration of 4 to 5 mM. The suspensions were incubated at 22 °C in the dark, and 20-μL portions were withdrawn at intervals, added to 2 mL of 10% trichloroacetic acid–50 mM sodium phosphate, and put on ice. These samples were washed on Millipore filters and radioactivity was determined by scintillation counting. The regeneration of rhodopsin by 11-*cis*-retinal in rod outer segment preparations, the extraction

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