LOCALISATION OF THE MAJOR SITE OF LIGHT STIMULATED PHOSPHORYLATION IN A REGION OF RHODOPSIN DISTINCT FROM THE CHROMOPHORE BINDING SITE

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1. Introduction

Approximately 80% of the protein of rod outer segments (ROS) is the visual pigment, rhodopsin, which consists of an apoprotein, opsin, and a chromophore, 11-*cis* retinal [1,2]. Several laboratories have established that in frog [3,4] and bovine [5–8] ROS rhodopsin is phosphorylated by ATP in a reaction which is stimulated by light. We have recently shown that ROS contain a specific protein kinase, 'opsin kinase' which catalyses the phosphorylation of bleached, but not unbleached, rhodopsin [8]. The requirement for a bleached substrate is considered to be due to the phosphorylation sites being rendered available to the kinase only after bleaching has induced conformational changes in rhodopsin [7,8]. In the present communication we describe experiments concerned with the localisation of the phosphorylation sites in rhodopsin.

The solubility of opsin kinase and the ease with which it can be extracted from rod outer segments indicates that it occurs either in the inter-discal space or is very loosely associated with the inter-discal surface of the disc membranes [8]. This would suggest that the region of rhodopsin which is phosphorylated is exposed on the inter-discal surface. In order to investigate this we have phosphorylated rhodopsin and then incubated the phosphorylated membranes with the proteolytic enzyme, papain. This enzyme removes some 36% of the polypeptide chain of rhodopsin from the inter-discal surface of the membranes leaving a membrane-bound core which contains all the spectral properties of the original visual pigment [9,10].

The present results show that the phosphorylation sites are in the part of the molecule digested by papain and that they are therefore located in a different region from the chromophore binding site and on the disc surface.

2. Methods and results

Rod outer segments were prepared from fresh dark-adapted bovine retinae as previously described [11]. They were then bleached by exposure to white light for 5 min and incubated at 37°C for 30 min in 50 mM Tris–HCl, pH 7.4, containing 1 mM $MgCl_2$ and 1 mM γ [^{32}P]ATP - conditions which result in the phosphorylation of all available protein sites [8].

After phosphorylation the membranes were collected by centrifugation at 100 000 *g* for 30 min and the pellet washed twice by resuspension and centrifugation in 67 mM sodium phosphate, pH 7.0.

The phosphorylated membranes were then

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suspended in 67 mM sodium phosphate buffer, pH 7.2, containing 5 mM cysteine and 2 mM EDTA, at a concentration of about 1 mg of protein/ml. The suspension was divided into three portions, one of which was saved, and the other two incubated at 37°C for 1 hr. At the beginning of the incubation period 1 part of papain (twice crystallised: obtained from Worthington) was added per 20 parts of ROS protein (w/w) to one of the two samples. After incubation all three samples were centrifuged at 100 000 *g* for 1 hr aliquots taken of the supernatants and the pellets. Trichloracetic acid was added to all samples to a final concentration of 7% (w/v) and the precipitated protein centrifuged down, washed and the bound radioactivity measured as previously described [8].

The results of this experiment are shown in table 1 from which it may be seen that only 18.3% of the protein bound phosphate was recovered in the insoluble material after papain digestion despite the fact that this material contained 64% of the rhodopsin protein and all of the bound retinaldehyde [9]. SDS gel electrophoresis of the insoluble material indicated that the radioactivity was associated with the small fraction of undigested rhodopsin rather than the material of mol. wt 24 800 which is the main insoluble product of the digestion [9]. In the control, where incubation was carried out in the absence of papain, some material was solubilised but 66% of the protein bound phosphate was found to be associated with the insoluble material. After papain digestion, nearly all of the radioactivity (74.2%) was in fact found in the soluble fraction but was not precipitated by trichloracetic acid. There would seem to be two possible explanations for this. Firstly, the radioactive phosphate could be present as inorganic orthophosphate released by the action of a phosphate during the incubation with papain. Secondly, it could be present as peptides too small to be precipitated by trichloracetic acid. To determine which of these possibilities is correct, samples of the supernatant were taken, made up to a volume of 1 ml, and 0.1 ml of 10 N perchloric acid added, followed by 0.3 ml of 5% ammonium molybdate and 2,5 ml of a mixture of isobutanol and benzene (1/1, v/v). The contents of the tubes were well mixed and briefly centrifuged to separate the two phases. Aliquots (2 ml) of the upper phase, which would contain inorganic phosphate [12] were taken and counted by Cerenkov radiation [13]. Only 4±1.5% (standard deviation of 6 observations) of the total radioactivity was found, so we can conclude that only a negligible amount of inorganic PO_4 was present in the supernatant after papain digestion. This was not increased if the acidified samples were heated at 100°C for 10 min to release inorganic phosphate from any

Table 1
Distribution of protein bound [^{32}P]phosphate following papain digestion of [^{32}P]phosphorylated rhodopsin.

Sample	Treatment	Proportion of radioactive phosphate present (percentage of amount present before incubation)	
		Incubated + papain	Incubated−papain
Insoluble material	trichloracetic acid precipitation	18.3 ± 0.9	66 ± 8
Soluble material	trichloracetic acid precipitation	1.7 ± 0.8	2.2 ± 0.7
Soluble material	none	74.2 ± 8.0	14.6 ± 2

Samples of cattle ROS were phosphorylated by incubation with [^{32}P]ATP and incubated, with or without papain, as described in the text. Samples of the original material and the soluble and insoluble fractions following digestion were either counted directly or precipitated, washed with trichloracetic acid and the radioactivity bound to the precipitated proteins determined as described in the text. The results are given as means ± standard deviations and are derived from 6 observations made in three separate experiments.

ATP not removed by the washing of the phosphorylated ROS before the papain digestion. These results suggest that the radioactive phosphate solubilised by the papain digestion was present as small peptides. To test this aliquots of the soluble fraction were taken and heated at 100°C for 6 hr with 2 M hydrochloric acid and phosphorylserine and phosphorylthreonine separated from the digests as described by Weller and Rodnight [14]. Some 30% of the total radioactivity was recovered at phosphorylserine and 12% as phosphorylthreonine, similar proportions being found in samples of the intact ROS before papain digestion. Since a breakdown of 50% of the phosphorylserine and phosphorylthreonine can be expected to occur during the acid hydrolysis [14], these observations clearly show that digestion of rhodopsin by papain releases nearly all the bound phosphate as a peptide or peptides which cannot be precipitated by trichloracetic acid and indicate that the major sites of rhodopsin phosphorylation are not on the insoluble material remaining after papain digestion even though this contains the retinaldehyde binding site.

As a further demonstration of this, an experiment was performed in which the insoluble material remaining after papain digestion was tested as a substrate for the 'opsin kinase' which can be extracted from ROS [8]. In this experiment ROS were digested with papain as described above but without prior phosphorylation. The insoluble fraction was centrifuged down at 100 000 *g* for 1 hr and washed twice by resuspension and centrifugation from 67 mM sodium phosphate buffer, pH 7.0, and finally incubated at a concentration of about 1 mg/ml in the presence of 50 mM Tris–HCl, 1 mM $MgCl_2$, 1 mM [^{32}P]ATP (specific radioactivity about 1 X 10^6 cpm/μmol) and about 20 μg/ml of a Tris extract prepared from ROS which contained the 'opsin kinase' activity [8]. The amount of radioactive phosphate transferred to protein was determined as previously described [8].

It may be seen from fig.1 that the insoluble material that is undigested by papain is a very poor substrate for opsin kinase although the tris extract used could catalyse the phosphorylation of partially purified rhodopsin (prepared by extraction of non rhodopsin-proteins from ROS with low concentrations of SDS as previously described [8]). It seems likely that the phosphorylation that does occur in the insoluble material is due to the presence of a small proportion of undigested rhodopsin.

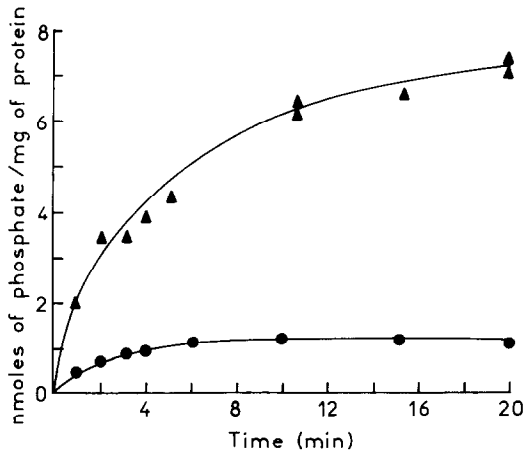

Fig.1. Inability of the insoluble material following papain digestion of rhodopsin to act as a substrate for 'opsin kinase'. Samples of the insolube material following digestion of ROS (●—●) or samples of partially purified rhodopsin (which had been incubated under exactly the same conditions as the papain treated ROS except that papain was not included in the mixture) (▲—▲) were incubated with [^{32}P]ATP and a soluble opsin kinase prepared from ROS [8] and the transfer of radioactive phosphate to the proteins determined as described in the text.

Discussion

The results reported here clearly show that the phosphorylation site(s) in rhodopsin are localised in a segment of the molecule which is digested (ultimately to small peptides) by papain. Our preliminary results also indicate that there may be only one site of phosphorylation in the molecule since only one major band containing radioactive phosphate could be detected following paper electrophoresis at pH 1.9 of the material solubilised by papain.

We have recently shown that bleaching of rhodopsin increases the Ca^{2+} permeability of the ROS discs while phosphorylation of the bleached molecules lowers the permeability to its dark value [15,16]. This suggests the possibility that the portion of the

rhodopsin molecule which is digested by papain and which contains the phosphorylation site may have an important role in controlling the permeability of the discs although it is distinct from the retinaldehyde binding site. Rhodopsin may, therefore, consist of two functionally distinct regions.

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