Monoclonal antibody ARC MAC 50.1 binds to a site on the phytochrome molecule which undergoes a photoreversible conformational change

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Monoclonal antibody from rat hybridoma cell line ARC MAC 50.1 binds more strongly to Pfr than to Pr in a sandwich ELISA of phytochrome in crude *Avena sativa* L. extracts or highly purified *Avena* phytochrome. Discrimination is not a consequence of differential modification of Pr and Pfr during the assay. Loss of a 6 kDa peptide from the N-terminal end of the phytochrome apoprotein during purification does not prevent discrimination. Neither is preferential binding to Pfr a consequence of a steric interaction between ARC MAC 50.1 and the antibody used with it in the sandwich ELISA. It is concluded that the binding site for ARC MAC 50.1 undergoes a reversible conformational change upon photoconversion and may thus represent a functional region of the phytochrome molecule.

Phytochrome *Monoclonal antibody* *Discrimination* (Avena) *Conformational change*

1. INTRODUCTION

Phytochrome is a regulatory chromoprotein ubiquitously distributed in higher plants. It exists in 2 forms called Pr and Pfr which are reversibly photo-interconvertible. Pr and Pfr can be distinguished by their light-absorbance characteristics; Pr showing maximal absorbance near 660 nm and Pfr at about 730 nm [1]. Biological action is believed to be a consequence of Pfr formation from Pr, whereas Pr is inactive. Photoconversion therefore acts as a biochemical switching mechanism to confer or remove the phytochrome molecule's capability for biological action. It is assumed that this reversible photo-activation of phytochrome is a consequence of a conformational change or changes in the phytochrome apoprotein which accompany photoconversion. Identification of regions involved in such changes is, therefore, an important step towards understanding the molecular mechanism of phytochrome action.

In a previous paper we showed that a panel of monoclonal antibodies (McAb) raised in rats to phytochrome, purified from dark-grown seedlings of *Avena sativa* L., contained some which reacted differentially with Pr and Pfr in ELISA of crude extracts of *Avena* and in partially purified phytochrome preparations [2]. Of particular interest was the McAb ARC MAC 50 which discriminated most strongly between Pr and Pfr and bound preferentially to Pfr. It is possible therefore that the binding site for this antibody is associated with a Pfr-specific conformation and hence binds to a possible functional region on the phytochrome molecule. Here we have investigated the reason for increased binding of ARC MAC 50 to Pfr. Evidence is presented that the differential reactivity of ARC MAC 50 with Pr and Pfr is the consequence of a photoreversible conformational change at its binding site and not an indirect result of either a Pr- or Pfr-dependent modification of

Abbreviations: PAGE, polyacrylamide gel electrophoresis; ELISA, enzyme-linked immunosorbent assay

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the protein or a conformational change elsewhere in the molecule.

2. MATERIALS AND METHODS

2.1. *Preparation of phytochrome samples*

Soluble extracts of phytochrome were prepared from 6-day-old dark-grown seedlings of *Avena sativa* L. cv. Saladin as in [2] and are referred to in the text as crude extracts. Purified phytochrome was a mixture of 114 and 118 kDa peptides having A_{660}/A_{280} = 0.7 prepared as described in [3]. Phytochrome concentration was calculated from $\Delta(\Delta A_{730-800})$ values using the extinction coefficient as determined by Roux et al. [4].

2.2. *Antibody preparations*

The production of rabbit anti-phytochrome IgG and monoclonal antibodies ARC MAC 50, 52, 54 and 56 has been described [2,3]. ARC MAC 50.1 was recloned in agar from ARC MAC 50 as in [5]. The McAb were partially purified from ascites fluid by 40% ammonium sulphate fractionation and stored in 20 mM phosphate buffer, 150 mM NaCl, pH 7.4 (PBS). Working dilutions were determined empirically. ARC MAC 50.1 was conjugated to alkaline phosphatase as in [3].

2.3. *ELISA for discrimination between Pr and Pfr*

Assays were carried out in darkness on 96-well microtiter plates (Nunc Immunoplate 1F, Gibco-Europe) and manipulations performed in dim green safe light. Plates were coated overnight at 4°C with 100 μl per well of either 4 $\mu g \cdot ml^{-1}$ rabbit antiphytochrome IgG or ARC MAC 56 in 0.05 M sodium carbonate buffer, pH 9.6. The following incubation steps were at 25°C and 3 washes with PBS containing 0.05% Tween 20 (PBS-Tween) given between each step. To each well was added 150 μl of 3% (w/v) BSA in PBS for 1 h, then 100 μl phytochrome as Pr or Pfr diluted in 1% (w/v) BSA in PBS-Tween (PBS-Tween-BSA) for 1.5 h. This was followed by 100 μl ARC MAC 50.1 or ARC MAC 50.1-alkaline phosphatase conjugate in PBS-Tween-BSA for 1 h. In the former case 100 μl rabbit anti-rat-alkaline phosphatase (Miles Laboratories, England) diluted 1:1000 in PBS-Tween-BSA was added for 1 h. Enzyme activity was determined using 1 $mg \cdot ml^{-1}$ *p*-nitrophenyl phosphate in 100 μl of 10% (v/v) diethanolamine-HCl, pH 9.8. Absorbance at 405 nm was determined with a Bio-Rad model 2550 EIA reader.

2.4. *SDS-PAGE and immunostaining*

Experiments were carried out as for initial steps of the ELISA, except that sample volumes were 2.5 ml and 70 × 11 mm Maxisorp tubes (Nunc, Gibco-Europe) were used rather than microtiter plates. Samples of Pr and Pfr were removed after 0 and 1.5 h incubation in the Maxisorp tubes and brought to 25 mM Tris, 1% SDS, 5% glycerol, 0.5% 2-mercaptoethanol, pH 6.8 (sample buffer), using sample buffer concentrated 10-fold, and heated at 100°C for 2 min. Following incubation with Pr and Pfr the tubes were washed 3 times with PBS-Tween and 0.5 ml sample buffer added. Bound protein was eluted by heating the tubes in a water bath at 80°C for 10 min with regular agitation. SDS-PAGE and electroblotting were carried out as described [6].

The immunostaining procedure was modified from [6] by including an additional 1 h blocking step in 10% goat serum and locating phytochrome peptides using a mixture of the anti-phytochrome McAb ARC MAC 50.1, 52 and 54, followed by rabbit anti-rat peroxidase (Miles Laboratories, England).

Light sources, red and far-red irradiation conditions were as described [2].

3. RESULTS AND DISCUSSION

To test for the possibility that discrimination in phytochrome extracts by ARC MAC 50.1 was a consequence of a Pr- or Pfr-dependent modification of the protein, the following experiments were performed. Crude plant extracts were pre-incubated as Pr or Pfr under the same conditions as in the ELISA and the ability of ARC MAC 50.1 to bind preferentially to Pfr in the subsequent assay was determined. As any chemical or proteolytic modification would be irreversible it would be expected to prevent discrimination between Pr and Pfr after subsequent photoconversions. The results showed that pre-incubation as either Pr or Pfr did not significantly affect discrimination between Pr and Pfr in the subsequent assay (fig.1A). Photoreversibility was retained following the pre-incubation. A similar pattern of reactivity was

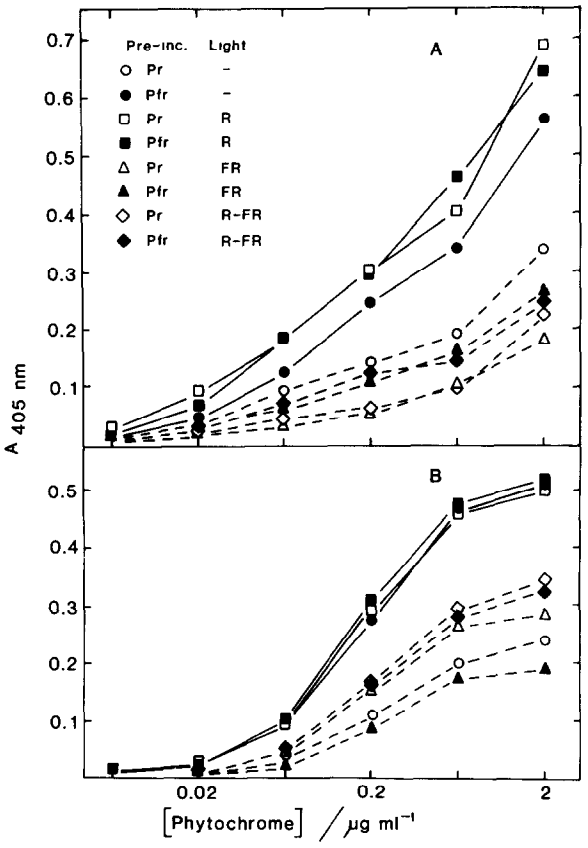

Fig.1. Effect of preincubation of phytochrome samples on discrimination between Pr and Pfr by ARC MAC 50.1 in ELISA. Dilution series of a crude phytochrome extract (A) or purified phytochrome (B) were irradiated with red light (R) or far-red light (FR) for 2 min to give Pr and Pfr, respectively. After pre-incubation in darkness at 25°C for 1 h samples were irradiated with R or FR in the sequence indicated and immediately assayed by ELISA using ARC MAC 50.1. Pre-inc., the form of phytochrome during pre-incubation; Light, irradiation treatment immediately prior to assay. (——) Samples assayed as Pfr, (– – –) samples assayed as Pr. Each point is the mean of 5 replicates, SE < 2%.

observed when the same experiment was carried out using highly purified phytochrome in which differential modification would not be expected (fig.1B). It has been reported that Pr but not Pfr is susceptible to proteolysis in crude extracts of *Avena* leading to the loss of a 6 kDa fragment from the N-terminal end of the molecule [7]. A further experiment was therefore carried out to test for selective proteolysis of the phytochrome during the ELISA. Pr or Pfr was incubated under the conditions of the ELISA but in plastic tubes to allow for the use of greater sample volumes. Phytochrome bound to the rabbit anti-phytochrome IgG during the incubation was eluted with SDS and separated by denaturing electrophoresis. Following electroblotting to nitrocellulose, phytochrome was located by immunostaining with a mixture of McAb (fig.2). When crude extract was tested, the predominant form of phytochrome was a 124 kDa species typical of undegraded *Avena* phytochrome, although a small amount of degradation occurred during the assay either as Pr or Pfr. This was despite the inclusion of PMSF which is reported to prevent Pr-specific proteolysis in crude extracts of *Avena* [7]. Crucially there was no indication of

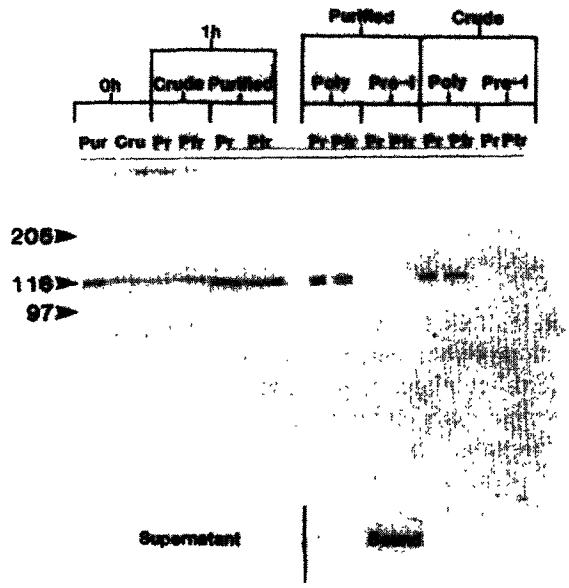

Fig.2. Immunostaining of phytochrome polypeptides bound out during ELISA as Pr or Pfr and of phytochrome remaining in the supernatant. Proteins were separated by SDS-PAGE and electroblotted onto nitrocellulose. Bands containing phytochrome were immunostained with a mixture of the monoclonal antibodies ARC MAC 50.1, 52 and 54. Pur, purified phytochrome; Cru, crude phytochrome extract; Poly, polypeptides bound to polyclonal rabbit anti-phytochrome; Pre-I, control using pre-immune rabbit IgG in place of rabbit anti-phytochrome. The position of molecular mass markers is indicated (205 = 205 kDa, etc.).

selective proteolysis of the phytochrome bound out by rabbit anti-phytochrome IgG or selective binding of Pr or Pfr. Purified phytochrome stained as a double band at a slightly lower molecular mass than phytochrome in the crude extracts. The mixture of 114 and 118 kDa peptides is typical of conventionally purified phytochrome [1]. Again, no evidence for selective modification or absorption as Pr or Pfr was observed. As discrimination between Pr and Pfr is observed for both crude and purified phytochrome preparations it cannot be due to the conformational change in the N-terminal region which allows proteolysis as Pr but not Pfr [7] which could only occur with intact phytochrome (i.e. crude preparations). This result is consistent with peptide mapping studies which have tentatively identified the binding site for ARC MAC 50.1 as being on the major chromophore-bearing domain but near the middle of the primary amino acid sequence [8,9].

We observe preferential binding of ARC MAC 50.1 to Pfr only if sandwich-type ELISAs are used. No discrimination is observed if purified Pr and Pfr are bound directly to ELISA plates (unpublished). This may explain the inability of others who have used this type of screen to identify discriminating antibodies [10]. Binding of Pr and Pfr directly to ELISA plates may lead to a loss of conformational differences between Pr and Pfr because of denaturation during binding.

A consequence of requiring 2 antibodies in combination for the assay is the possibility that discrimination could arise interactively through steric factors. Thus conformational changes distant from the antibody binding sites leading to changes in the positions of the antibodies relative to each other could lead to apparent discrimination. To minimize this possibility a polyclonal antibody preparation which should bind phytochrome in a randomly orientated manner was used routinely in the screen for discriminating antibodies [2]. It is feasible, however, that the antibodies in the polyclonal preparation are predominantly directed at a single antigenic region on the phytochrome molecule, in which case steric factors could be significant. ARC MAC 50.1 was therefore also tested for its ability to discriminate between Pr and Pr bound to ARC MAC 56, a non-discriminating McAb [2]. ARC MAC 56 was chosen because it showed no interaction with

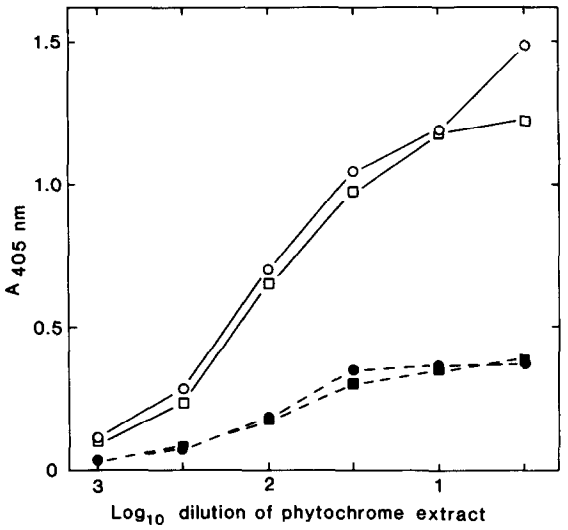

Fig.3. Effects of replacing rabbit anti-phytochrome IgG with monoclonal antibody ARC MAC 56 on discrimination by ARC MAC 50.1 between Pr and Pfr in ELISA. (□, ■) Rabbit anti-phytochrome, (○, ●) ARC MAC 56, (■, ●) Pr, (□, ○) Pfr. Each point is the mean of 4 replicates, SE < 3%.

polyclonal antibodies or ARC MAC 50.1 in competition assays and gave the strongest signal of all our McAb when used in combination with polyclonal antibodies in ELISA. The binding site for ARC MAC 56 must, therefore, be different from the predominant binding sites for the polyclonal antibodies. Assays in which the discrimination between Pr and Pfr was compared for ARC MAC 50.1 in combination with ARC MAC 56 and polyclonal antibodies gave almost identical results (fig.3). Discrimination is therefore independent of the antibody used in combination with ARC MAC 50.1. The conclusion of these experiments is that ARC MAC 50.1 itself binds to a site which is subject to a conformational change during photoconversion. It binds more strongly to Pfr, raising the possibility that the site is associated with some functional property of the molecule. No interaction between ARC MAC 50.1 and the spectral properties of Pr or Pfr has been detected in our laboratory. This is consistent with the apparent lack of requirement for the N-terminal cleavable peptide, which interacts closely with the chromophore [1], for discrimination by ARC MAC 50.1. Proteolytic studies have indicated that

conformational changes in a number of regions of the phytochrome molecule accompany photoconversion [11,12]. Some of these are distant from the chromophore. It is possible to envisage that the light-absorbing and regulatory functions of phytochrome are invested in different parts of the molecule. If this is the case, discriminating antibodies such as ARC MAC 50.1 will be invaluable in the location of parts of the phytochrome molecule with regulatory functions.

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