Volume 234, number 2, 374–378 FEB 06077 July 1988

The interaction between the 33 kDa manganese-stabilising protein and the D_1/D_2 cytochrome *b*-559 complex

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Received 26 April 1988

Using affinity chromatography with the extrinsic 33 kDa protein as the immobilised ligand, it was demonstrated that the reaction centre complex of photosystem II, composed of the D_1, D_2 and cytochrome *b*-559 polypeptides, can directly interact with the 33 kDa protein. By this approach it was possible to purify the reaction centre from solubilised photosystem II core complexes since neither the 47 kDa nor the 43 kDa protein would bind to the ligand.

Reaction center complex; Manganese-stabilizing protein; Oxygen evolution; Affinity chromatography; Photosystem II

1. INTRODUCTION

A number of recent studies have given experimental support to the idea that the D_1 and D_2 polypeptides of PSII form a reaction centre complex similar to that found in purple photosynthetic bacteria. Nanba and Satoh [1] were the first to isolate a chlorophyll-binding complex consisting of the D_1 and D_2 polypeptides and the α- and β-apoproteins of cytochrome *b*-559 (products of chloroplast genes, *psb*A, *psb*D, *psb*E and *psb*F, respectively). We confirmed and extended their findings [2,3]. This and other work [4,5] have indeed shown that the isolated D_1/D_2/cyt *b*-559 complex contains the primary donor P680 and the primary acceptor pheophytin. For reasons which are not yet clear this isolated PSII reaction centre complex does not bind the plastoquinones which constitute Q_A and Q_B. Recent studies, however, have revealed important knowledge about the oxidising side of P680. It now seems very likely that the immediate electron donor to $P680^+$, called Z, is in fact a tyrosine on the D_1 polypeptide, probably at position 160 [6,7]. The close proximity of Z to P680 within the D_1/D_2 complex would account for the rapid rate of electron transfer between the two species [8]. These new findings raise questions about the minimal unit required for electron flow from water to P680 and the location of the Mn custer. It would now seem likely that the Mn cluster involved in water splitting is very closely associated with the exposed portions of the D_1 and D_2 polypeptides on the lumenal side of the thylakoid and, indeed, speculative models along these lines are emerging [9,10]. If this is so questions are raised concerning the proximity and perhaps direct interaction of the 33 kDa extrinsic protein with the reaction centre complex. The 33 kDa extrinsic protein seems to be vital for the water splitting process and plays a role in stabilising the Mn cluster [11,12]. The role of the other two chlorophyll-binding proteins found in PSII cores, namely CP47 (product of the chloroplast *psb*B gene) and CP43 (product of the chloroplast *psb*C gene) in binding the 33 kDa protein should also now be questioned. In this communication we report the use of an affinity column with the 33 kDa protein as the ligand to investigate the interaction between the 33 kDa protein and the PSII core polypeptides. Our results indicate that there is a specific interaction between the 33 kDa and the

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Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/88/$3.50

PSII reaction centre consisting of D_1/D_2/cyt *b*-559 polypeptides but not the CP47 or CP43 components.

2. MATERIALS AND METHODS

Photosystem II enriched membranes were prepared from pea *(Pisum sativum)* thylakoids according to the method in [13] and stored at −80°C. Using these membranes purified oxygen evolving PSII core complexes were prepared by the use of β-octylglucoside essentially as described in [14]. PSII reaction centre complexes were isolated as detailed in [2,15]. The extrinsic 33 kDa protein was purified from PSII enriched membranes either by the procedure involving phase partitioning with butanol [16] or by 1 M $CaCl_2$ washing of membranes previously subjected to 1 M NaCl washing in order to remove the 23 and 16 kDa peripheral proteins. In both cases the purified protein was found to be electrophoretically pure.

Affinity chromatography was carried out using CNBr-activated Sepharose 4B onto which the isolated 33 kDa protein was coupled. Prior to the immobilisation, solutions of this latter protein were extensively dialysed against distilled H_2O at 4°C. The final solution was freeze-dried and the resultant protein was resuspended in 0.1 M $NaHCO_3$, pH 8.3, containing 0.5 M NaCl (coupling buffer). The immobilisation of the 33 kDa protein is described below. Dried CNBr-activated Sepharose 4B was washed and reswollen in 1 mM HCl. The gel was collected by centrifugation at $2000 \times g$ for 10 min at 4°C, resuspended in coupling buffer and mixed for 30 min at room temperature. The gel was collected again by centrifugation as above and resuspended in the 33 kDa protein solution. The suspension was mixed overnight at 4°C, the gel recovered by centrifugation and the supernatant containing the unbound 33 kDa protein was retained. Remaining active groups on the gel were blocked by incubation with 0.2 M glycine, pH 8.0, and mixing overnight at 4°C. Any excess adsorbed protein was washed away by sequentially washing the gel with 0.1 M Na acetate, pH 4.0, containing 0.5 M NaCl and with coupling buffer. Finally, the protein-Sepharose conjugate was resuspended in coupling buffer. Under these conditions coupling efficiency varied from about 30 to 50% and the coupling was estimated by determining the 33 kDa protein concentration in the original solution (absorbance at 276 nm with extinction coefficient of 20 mM^{-1} of [17]) and in the various supernatants collected after coupling to the gel. The 33 kDa protein-Sepharose conjugate was packed into a column (3 cm × 1 cm) and equilibrated with 20 mM Mes, pH 6.5, containing 0.2% Triton X-100 (running buffer).

PSII core complexes prepared as in [14] were treated further with 1 M $CaCl_2$ in 20 mM Mes, pH 6.5, in order to remove the 33 kDa protein associated with the complex. The pellet resulting from a centrifugation at $40\,000 \times g$, 4°C, for 20 min was further solubilised by resuspending in 20 mM Mes, pH 6.5, to which Triton X-100 was added to a concentration of 1% with a final chlorophyll *a* concentration of 100 μg/ml. The sample was stirred on ice in the dark for 30 min and then diluted 5-fold with 20 mM Mes, pH 6.5. This solution was loaded onto the affinity column at 0.4 ml/min and the column was washed with running buffer until the eluant was colourless and no absorbance at 280 nm was recorded. Bound polypeptides were eluted with 1 M $CaCl_2$ in running buffer, at 0.2 ml/min.

Purified D_1/D_2/cyt *b*-559 complex (usually with a chl *a* concentration of 130–180 μg/ml) was diluted to 5 μg/ml with running buffer, loaded onto the affinity column and treated as above.

SDS-PAGE was carried out in 10–17% acrylamide gradient gels containing 6 M urea and run at 12°C. Immunoblotting was carried out by electrophoretic transfer of the proteins separated by SDS-PAGE onto nitrocellulose (pore size 0.2 μm) as described in [18]. After the transfer the nitrocellulose was 'blocked' in a medium containing 50 mM Tris, pH 7.4, 150 mM NaCl and 5% skimmed milk for 5 h and then probed overnight with antisera against the *psb*A (D1) and *psb*D (D2) products [19]. Visualisation of bound antibody was achieved by incubation with goat anti-rabbit IgG-alkaline phosphatase conjugate followed by the addition of the substrates 5-bromo-4-chloro-3-indolyl-phosphate *p*-toluidine salt (BCIP) and nitroblue tetrazolium chloride (NBT) in a buffer containing 100 mM Tris, pH 9.5, 100 mM NaCl and 50 mM $MgCl_2$.

Cytochrome *b*-559 content was estimated by recording the oxidised (ferricyanide) minus reduced (dithionite) spectra and using an extinction coefficient of 15 mM^{-1} at 570–559 nm [20].

3. RESULTS

The room temperature absorption spectrum of the PSII core complex, washed with 1 M $CaCl_2$ and further solubilised with Triton X-100, is shown in fig.1A. There is an absorption maximum at about 672 nm in the red end of the spectrum and an absorption maximum at 435 nm followed by a shoulder at 415 nm in the blue. The chl *a* to cytochrome *b*-559 ratio of this preparation was estimated to be 26.5. When the solubilised PSII core preparation was applied to the 33 kDa affinity column a fraction remained bound and was eluted with 1 M $CaCl_2$. The absorption spectrum of this $CaCl_2$ eluted fraction is shown in fig.1B. Compared with fig.1A there is a distinct difference in the blue region of the spectrum as the absorption maximum is now at 415 nm and there is a shoulder at 435 nm. The chl *a* to cytochrome *b*-559 ratio of this fraction was estimated to be 5.9 indicating a 4–5-fold enrichment of this cytochrome in comparison with the starting material. The spectrum shown in fig.1B as well as the chl *a* to cytochrome *b*-559 ratio of this preparation are very similar to those obtained from D_1/D_2/cyt *b*-559 complex [1,2].

Fig.2A shows the absorption spectrum of the PSII reaction centre complex composed of the D_1, D_2 and cytochrome *b*-559 polypeptides which was applied to the affinity column. A proportion of

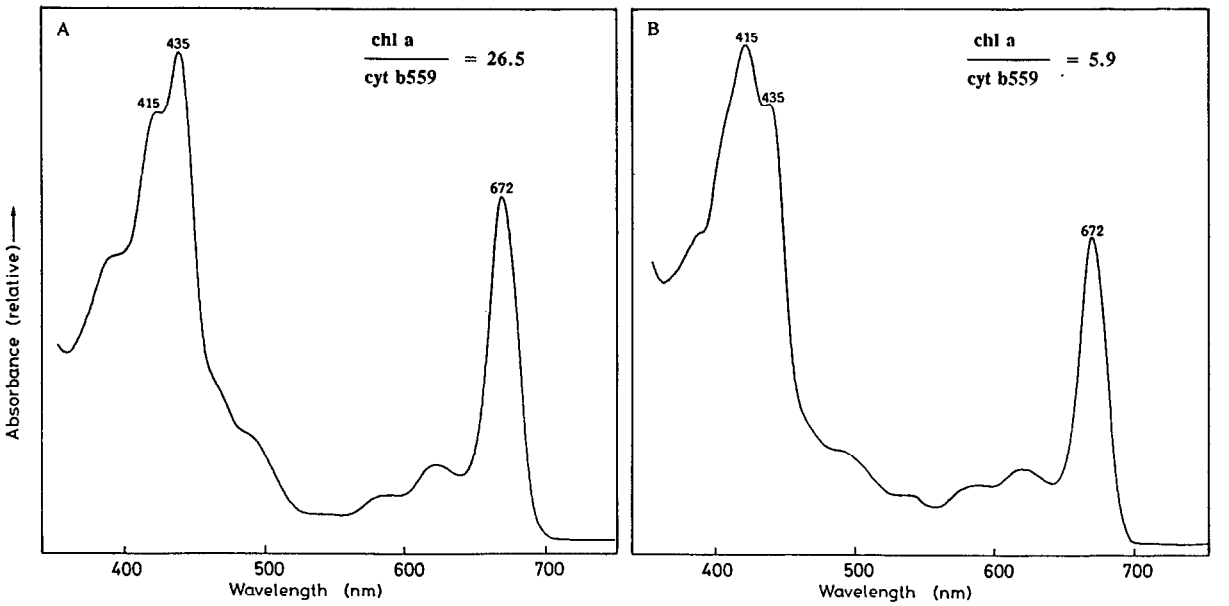

Fig.1. Room temperature absorption spectra of (A) the PSII core complex after solubilisation with Triton X-100 and (B) of the complex retained by the 33 kDa-affinity column.

this preparation was retained by the column under the conditions used and the absorption spectrum of the retained complex is shown after elution in fig.2B. The red absorption maximum was shifted to 674 nm and the ratio of the 415 nm to 435 nm absorptions was increased. The chl *a*:cytochrome *b*-559 ratio was reduced to 4.1 in the complex retained by the 33 kDa protein affinity column as

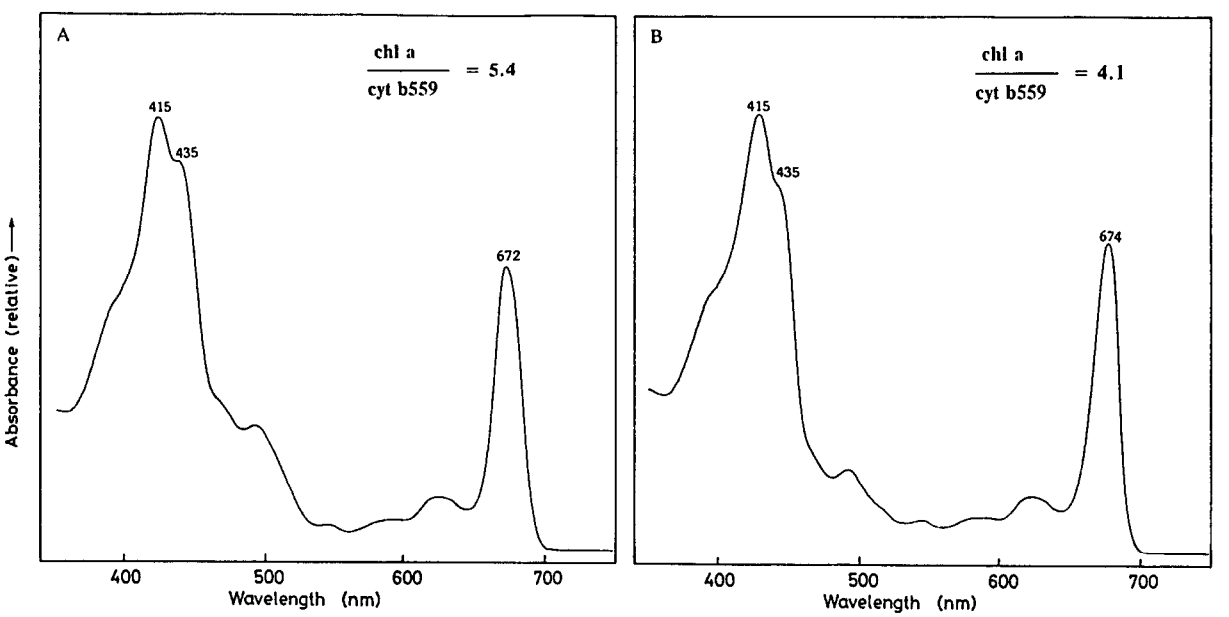

Fig.2. Room temperature absorption spectra of (A) the PSII reaction centre complex and (B) the complex retained by the 33 kDa-affinity column.

compared with the ratio of 5.4 in the starting material, indicating a further enrichment of cytochrome *b*-559 content. We presume that this reflects the loss of contaminating chl *a* present in the starting material as well as a selective removal of a population of the reaction centre complexes.

SDS-PAGE of the complexes used in this study is shown in fig.3A. The PSII core complex (lane 1) is composed of essentially five polypeptides; the CP47, CP43, D_1, D_2 and the polypeptides of the cytochrome *b*-559 (the 4 kDa polypeptide of the cyt *b*-559 apoprotein is not resolved in the gel shown). The PSII reaction centre composed of the D_1, D_2 and cyt *b*-559 is shown in lane 3. Coomassie stained bands at molecular masses above 50 kDa have been attributed to aggregated forms of D_1 and D_2 [1,3]. Lanes 2 and 4 show the polypeptide composition of the particles retained by the 33 kDa affinity column from the Triton solubilised PSII cores and the PSII reaction centres, respectively. It is apparent that both types of particle are indistinguishable in terms of their polypeptide composition. There are two Coomassie stained bands in the region of 30–32 kDa and one at about 9 kDa. An aggregated protein complex is also observed at a higher molecular mass. We have confirmed the identity of these proteins by immunoblotting using antisera raised against the *psb*A (D_1) and *psb*D (D_2) gene products. Fig.3B shows that the protein band at about 30 kDa cross-reacted with the antibodies against the *psb*A gene product and Fig.3C indicates the cross-reactions of the *psb*D gene product with the 32 kDa protein.

A column prepared without the 33 kDa protein but employing 0.2 M glycine for binding to active groups did not retain the PSII reaction centre. In a control experiment it was shown that purified and further solubilised LHCP also did not bind to the 33 kDa affinity column indicating that the interaction was not non-specific. On the other hand, we found, as did Isogai et al. [21], that the 33 kDa affinity column does bind the unsolubilised PSII core preparation. Although we routinely eluted the reaction centre complex from the column with 1 M $CaCl_2$ we also observed that the bound complex could be released with an elution buffer containing 1 M NaCl and 0.2% Triton X-100. Normally high concentrations of NaCl do not remove the 33 kDa protein from PSII [12] but we have found that the inclusion of 0.2% Triton X-100 in the washing

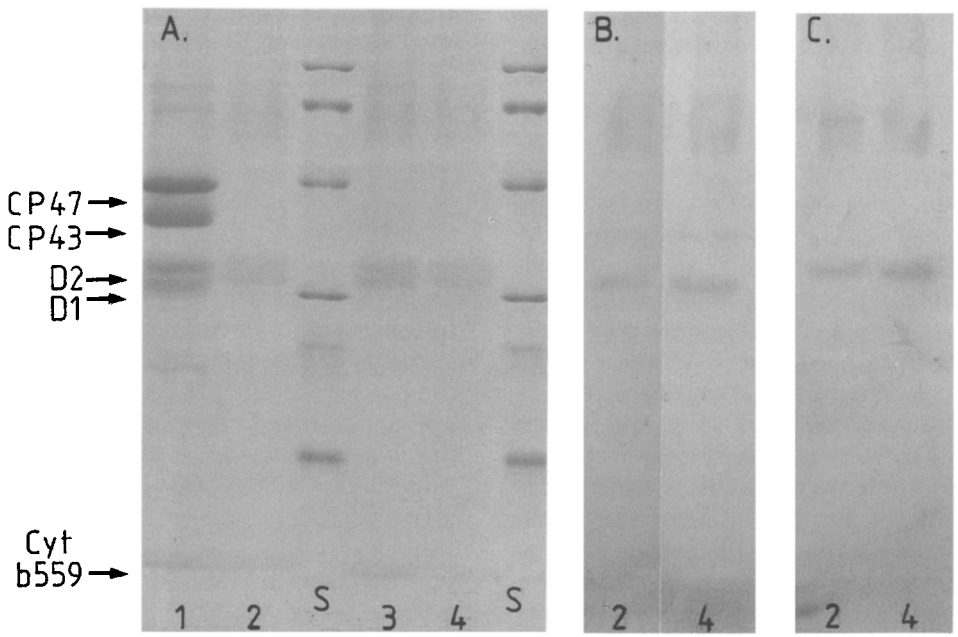

Fig.3. (A) SDS-PAGE of the $CaCl_2$-washed PSII core complexes (lane 1), and the PSII reaction centre complex (lane 3). The fractions interacting with the 33 kDa-affinity column when PSII cores or PSII reaction centres were used as the starting material are shown in lanes 2 and 4, respectively. Samples as those shown in lanes 2 and 4 were subjected to immunoblotting with antibodies against the *psb*A (D_1; B) and *psb*D (D_2; C) gene products. S, molecular mass standards.

medium does, for example, allow 1 M NaCl to remove this extrinsic protein from isolated PSII core particles (not shown).

4. DISCUSSION

The fact that the affinity column could selectively remove the PSII reaction centre consisting of D_1/D_2/cyt *b*-559 from the solubilised PSII cores, as well as bind the isolated D_1/D_2/cyt *b*-559 preparation, suggests that there is a specific interaction between the 33 kDa manganese-stabilising protein and the D_1/D_2/cyt *b*-559 reaction centre complex. We found that neither the 47 kDa nor the 43 kDa chlorophyll *a* binding proteins interact with the 33 kDa polypeptide under the conditions of our experiments. However, when the isolated PSII core complex was not treated with Triton X-100 the complex containing the 47 kDa and 43 kDa was retained on the column. This latter result is consistent with the work of Isogai et al. [21] who too were able to bind a PSII core particle, composed of polypeptide with apparent molecular masses of 47, 43, 32, 30 and 8 kDa to an affinity column incorporating the 33 kDa protein as the ligand. It must therefore be concluded that the retention of the PSII core on the affinity column is due to interactions between one or more of the polypeptides which constitute the D_1/D_2/cyt *b*-559 complex and not with the 47 or 43 kDa polypeptides.

Recently it has been reported [22] on the basis of crosslinking experiments employing bifunctional reagents, that the 47 kDa protein must be in close proximity and perhaps in direct association with the 33 kDa protein. It has also been suggested [23] on the basis of protease digestion experiments that the 33 kDa protein is associated with the 43 kDa protein, since the latter protein was more accessible to digestion after the removal of the 33 kDa polypeptide. The above experiments, however, provide no direct evidence that there is a specific interaction between the 33 kDa manganese-stabilising protein and either of the chlorophyll *a* binding antenna proteins.

Although our data indicate that there is specific interaction between the 33 kDa and the D_1/D_2/cyt *b*-559 reaction complex it does not rule out the possibility that other proteins are required in order to establish the correct conformation of the 33 kDa in relation to its role in stabilising the Mn cluster and catalysing the oxidation of water. These other proteins may be required to confer the correct environment necessary for the water splitting process to proceed.

Acknowledgements: We acknowledge financial support from the Agricultural and Food Research Council and valuable technical assistance from Jenny Nicolson, John DeFelice and Kenneth Davis.

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