Estimation of the functional size of Photosystem II

J.H.A. Nugent* and Y.E. Atkinson

Department of Botany and Microbiology, University College, Gower Street, London WC1E 6BT, England

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The radiation inactivation technique was used to measure the functional size of Photosystem II from *Phormidium laminosum*. Using frozen samples irradiated in liquid nitrogen, the technique was shown to give reproducible results. The mean functional size of PS II was found to be 125 kDa in a range from 99 kDa to 160 kDa. Comparing this result to those reported in other studies using SDS–PAGE suggests this functional size could be composed of one 52-kDa reaction centre polypeptide, one 33-kDa herbicide binding protein, and one 34-kDa polypeptide associated with oxygen evolution. Even the maximum limit of the estimate would only allow the further inclusion of approximately 40 kDa of polypeptide. The result suggests a smaller and simpler composition for PS II than indicated by SDS–PAGE.

Phormidium laminosum *Photosystem II* *Photosynthesis* *Oxygen evolution* *Radiation inactivation*

1. INTRODUCTION

Photosystem II (PS II) is a membrane-bound protein complex found in the chloroplasts of higher plants, algae and cyanobacteria. PS II converts the light energy absorbed by the pigment array to chemical energy. It achieves this using two electron transfer chains joined by the reaction centre chlorophyll (Chl), P680. One chain transfers electrons from the photooxidised P680 to an iron-quinone acceptor, producing reduced quinone. The second chain reduces photooxidised P680 using electrons from water. The sequential removal of 4 electrons from water by 4 turnovers of PS II leads to the production of oxygen. The electron acceptors of the first chain are similar to those of purple photosynthetic bacteria, but the second chain, the oxygen evolving system (OES), is unique to PS II.

A number of partially purified PS II preparations which retain high rates of oxygen evolution have been described. The preparation from the thermophilic cyanobacterium *Phormidium laminosum* was the first to be isolated and its polypeptide and electron transfer components have been studied [1–6]. The mechanism of electron transfer, especially from water to P680, is only partially known. SDS-polyacrylamide gel electrophoresis (SDS–PAGE) of PS II preparations including *P. laminosum* [4] has led to several polypeptides being assigned to PS II. However, the total number of polypeptides, their stoichiometry, organization and electron transfer function, are not known.

Radiation inactivation is a proven technique for measuring the functional molecular size of both soluble and membrane-bound proteins [7–10]. The advantages of this technique include: (i) the ability to measure membrane proteins in situ, using crude preparations; (ii) the technique gives the functional size of the complex, rather than the relative molecular masses of individual polypeptides.

We here report the results of experiments using a PS II preparation from *P. laminosum* to evaluate the radiation inactivation technique for use on photosynthetic complexes. The functional size

* Present address: The Ciba Foundation, 41 Portland Place, London W1N 4BN, England

Abbreviations: DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; DMBQ, 2,6-dimethyl-1,4-benzoquinone; ESR, electron spin resonance; NAD, nicotinamide-adenine dinucleotide

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of PS II is compared to the relative molecular masses of polypeptides assigned to PS II by other techniques.

2. MATERIALS AND METHODS

Photosystem II from *P. laminosum* was prepared as in [1,5]. Preparations giving control values of between 500 and 1500 μmol $O_2 \cdot$ mg $Chl^{-1} \cdot h^{-1}$ were used. Samples (0.2 ml) of the PS II preparation at approximately 1 mg Chl $\cdot$ ml^{-1} were placed in 0.3-cm diameter ESR tubes and then frozen in liquid nitrogen. Yeast alcohol dehydrogenase (alcohol:NAD^+ oxidoreductase, EC 1.1.1.1) (Sigma) was used for molecular mass calibration. The enzyme was mixed with the PS II prior to sample preparation at a concentration of 20 units $\cdot$ ml^{-1}.

Samples were stored in liquid nitrogen and were kept frozen by packing with solid carbon dioxide pellets during transportation.

Irradiation was performed with the MEL SL 75/20 linear accelerator at Addenbrooke's Hospital, Cambridge. A uniform 16 MeV beam, 10 cm in diameter, was produced by a diffuser screen. For each radiation dose, two samples were placed in a finger dewar containing liquid nitrogen. The dewar was held in the beam surrounded by a lead target block which was cooled by air from a fan. Calibration of the radiation dosage at the target block was routinely performed by using the perspex absorbance method [11]. Dosage was maintained at 2 Mrad $\cdot$ min^{-1} until the integrated radiation dosage was achieved. The requirement for irradiation at 77 K is discussed below.

Oxygen evolution was measured at pH 7.5 as in [5], with the average initial rate from duplicate or triplicate assays being recorded. Care was taken to ensure that light intensity and the concentration of electron acceptors, DMBQ and ferricyanide were saturating. The amount of sample used was identical within the same series of assays, but varied from 3–10 μg Chl $\cdot$ ml^{-1} between experiments. The sensitivity of oxygen evolution to DCMU was checked on each set of samples by measurements in the presence of 5 μM DCMU.

Alcohol dehydrogenase (ADH) activity was measured in a freshly made solution containing 60 mM glycine, 50 mM semicarbazide, 0.03% ethanol and 1 mM β NAD at pH 8.9; 1–5 μl of sample was added to 1.2 ml of this solution and the ADH activity recorded as the initial rate of production of NADH. NADH production was measured as the increase in absorption at 340 nm, using a Cary G12 spectrometer and matched quartz cuvettes.

2.1. *Target theory*

The high energy radiation used in this study deposits 60–100 eV per hit and target theory [7,12] assumes that this primary reaction causes covalent bond breakage, severe damage to the polypeptide structure and loss of function. Inactivation is limited to individual peptide chains. Energy transfer does not occur between non-covalently linked molecules and is also inefficient between the polypeptide backbone and lipid or carbohydrate linked to amino acid side chains [9,10,12,13].

The probability of a hit on a particular polypeptide has been developed into an empirical equation [7]. This is shown in eq.1:

$$\frac{6.4 \times 10^5}{D_{37}} = \text{molecular size in Da} \qquad (1)$$

where D_{37} is the dose in Mrads required to reduce the activity of the sample to 37% of control values. This equation applies to irradiation at room temperature. Radiation sensitivity decreases as the temperature is reduced and temperature correction is required. Ideally, samples should be lyophilised before irradiation in order to minimise the possibility of secondary reactions occurring via reactive oxygen intermediates. However, as an alternative, frozen samples can be used as this restricts the mobility of radicals. PS II preparations from *P. laminosum* were inactivated by lyophilisation, but activity was retained after freezing and thawing. Therefore frozen samples were used in the series of experiments reported here. PS II activity was defined as the initial rate of oxygen evolution. Electron transport was presumed to occur from water via P680 and secondary quinone acceptors to the artificial electron acceptors. The sensitivity of oxygen evolution to DCMU was assumed to indicate the degree of involvement of secondary quinone acceptors.

Each measurement (A) was converted to a percentage of the unirradiated control value. A_0 (the calculated activity at zero dose) was obtained by linear regression on a plot of log A *vs* dose, with

all data points equally weighted. From this the slope of the regression line and activity at 0 Mrad were calculated from:

$$\log\left[\frac{A}{A_0}\right] = -kD \qquad (2)$$

where D is the radiation dose, and k is the constant proportional to the slope of the line and the molecular size of the target protein. The molecular mass used for yeast ADH [7,14] was 141 kDa. Calculation of the D_{37} for ADH in liquid nitrogen was used to determine the temperature correction factor by comparing the value obtained with that calculated for room temperature using eq.1.

3. RESULTS AND DISCUSSION

The radiation sensitivity of PS II from *P. laminosum* was tested in a series of 4 experiments. In each experiment PS II activity decayed as a single exponential function of radiation dose. Analysis of the results from these individual experiments gave D_{37} values (with standard deviations) in Mrad of 19 ± 2, 23 ± 2, 24 ± 1.5 and 19 ± 1.5. Fig.1 shows the 4 experiments presented as a single plot of log activity *vs* radiation dose. This shows a simple dose–response relationship up to the highest dose used. The D_{37} is 21 ± 2 Mrad (38 readings). The differences between experimental results occur from a variety of factors in addition to the normal experimental error from the assay. These include: (i) variation in the strength and calibration of the radiation beam; (ii) the possibility of secondary reactions tending to increase inactivation rate; (iii) variation of quenching effects between samples. However, as shown by fig.1, the results are reproducible to reasonable accuracy.

The radiation sensitivity of frozen samples irradiated in liquid nitrogen is lower than that of lyophilised samples irradiated at room temperature. A recent study [15] has demonstrated a linear relationship between temperature and radiation dose, confirming earlier indications. The study in [15] suggests that the temperature factor between liquid nitrogen and room temperature will be approximately 4. In order to test this and provide a calibration of the radiation inactivation technique, yeast alcohol dehydrogenase was added to PS II samples in two experiments and its activity was assayed following irradiation. The ADH activity decayed as a single exponential function of radiation dose giving a D_{37} of 18.7 ± 2.5 Mrad (13 readings). Radiation inactivation studies of yeast ADH give the functional size of ADH as the total molecular mass [8,12], of its 4 identical subunits. Therefore, using the molecular mass of ADH (141 kDa), and eq.1, the calculated temperature correction factor in this study was 4.1. The standard deviation on the ADH D_{37} value gave the range as between 3.6 and 4.7. This result is therefore close to that expected.

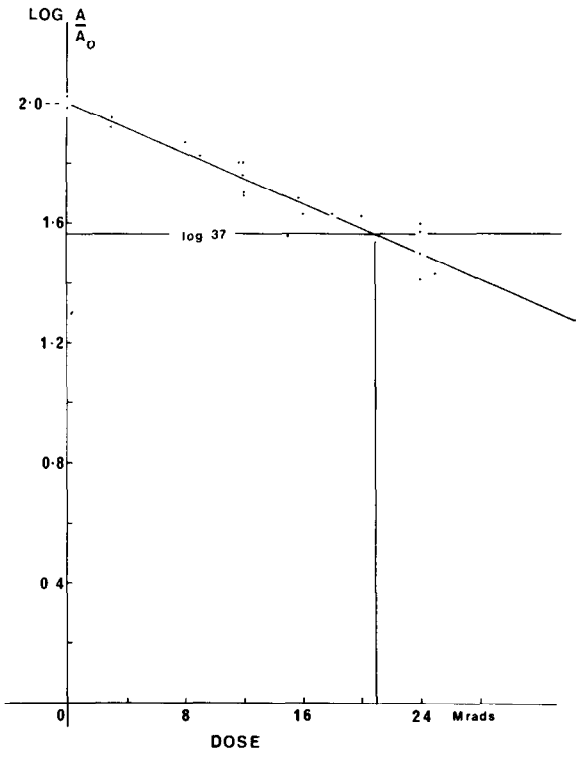

Fig.1. Relationship between PS II activity and radiation dose. A/A_0 is the PS II activity (oxygen evolution) relative to that (A_0) prior to irradiation, plotted on a logarithmic scale. All data points are means of either duplicate or triplicate assays with the line fitted by regression analysis. The intersect at 37% activity which gives the D_{37} dose value is shown.

The calibration of radiation inactivation by ADH enables the molecular size of PS II activity to be calculated. This can be done either by comparison of the D_{37} values for both ADH and PS II activities or by comparing the slopes of their log A *vs* dose regression lines.

The functional size of PS II from this comparison is 125 kDa. At the highest dose levels oxygen evolution was 30–40% of control values and therefore it can only be assumed that 60–70% of PS II complexes, i.e., those inactivated, have this functional size. The standard deviation on both ADH and PS II D_{37} values means that maximum and minimum values for the functional size are 160 and 99 kDa, respectively.

Apart from PS II and ADH assay, the main error would arise from secondary reactions involving oxygen radicals. The effect of these are to reduce the D_{37} value, which in turn increases the estimate of functional size (eq.1). Irradiated samples produced foam on thawing, and controls did not, indicating possible secondary effects. Addition of 5 mM sodium ascorbate or 0.5 mM dithiothreitol to samples prior to irradiation resulted in a greater rate of inactivation with increasing dose than found with untreated controls. This suggests that these additions increased secondary inactivation. The functional size obtained for PS II does, however, suggest that in untreated samples secondary effects were slight.

A number of polypeptides have been assigned to PS II by SDS–PAGE of samples from a variety of preparations. Major polypeptides include two associated with the reaction centre, of approximately 50 and 45 kDa [16,17]. The 50-kDa polypeptide probably contains P680 [18]. A 34-kDa polypeptide is suggested to be associated with water oxidation and manganese binding [17,19–21]. A 32-kDa polypeptide is associated with the binding of secondary quinone electron acceptors and is also the binding site of many herbicides, including DCMU [22]. Two further polypeptides, of approximately 24 and 16 kDa, can be removed from the oxidising side of PS II by various treatments such as salt washing or high pH [17,23] and finally the apoprotein of cytochrome *b*-559, a component also on the oxidising side of PS II has been assigned to a 10-kDa band [24]. In addition to these 7 major polypeptides, a number of light-harvesting proteins as well as a further range of polypeptides, probably contaminants, have been reported in PS II preparations. Recent improvements in the purification of PS II from *P. laminosum* [4] give a preparation which has bands of 52.4, 43.2, 33.9, 32.9 and 30 kDa assigned to PS II. No evidence was seen for a 24-kDa component and no band was assigned to cytochrome *b*-559, although stained bands were present in the 10-kDa region.

Clearly the functional size estimate of 125 kDa produced by the study cannot include all these polypeptides. Indeed, errors due to secondary reactions may have increased the size estimate. The size should include the 32.9-kDa herbicide binding protein, as the PS II activity was DCMU-sensitive up to the highest radiation dose. It should also include both the 52-kDa reaction centre polypeptide, which probably contains P680, and the 34-kDa protein associated with oxygen evolution. Assuming a 1:1:1 ratio for these proteins, there is only room within the maximum limits of the estimate for a small polypeptide, perhaps the 10-kDa cytochrome *b*-559 and/or the 30-kDa polypeptide. The assay conditions should preclude any involvement of light-harvesting proteins, which possibly include the 43.2-kDa polypeptide in the functional size. The apparent absence of the 24-kDa polypeptide in PS II preparations from *P. laminosum* allows a more straightforward analysis than would be possible on the PS II from higher plants, where removal of this protein has been shown to affect rates of oxygen evolution [25].

The result of this study is therefore a smaller and simpler estimate of the functional size of PS II than expected. Further experiments using radiation inactivation techniques on both PS II and other photosynthetic protein complexes will provide an interesting comparison and may improve the accuracy of the results presented here.

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