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Radiation inactivation studies on Photosystem I. Functional sizes of electron-transport reactions

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Radiation inactivation studies on the functional size of electron-transport processes in the Photosystem I reaction-centre complex showed the following characteristics. (1) The molecular mass required for electron transport from P-700 to iron-sulphur centre A was below 40 kDa. (2) Independent inactivation of iron-sulphur centres A and B was observed indicating their location on separate polypeptides. (3) The molecular mass of the polypeptides containing iron-sulphur centres A and B were 5–10 kDa based on a linear electron-transfer chain or 15–20 and 5–10 kDa (centre B) based on a branched chain. (4) A reaction centre 'core' containing the electron carriers for electron transport from P-700 to iron-sulphur centre X was indicated. These observations are discussed in comparison to current ideas on the polypeptide composition of the Photosystem I reaction centre. It is concluded that the radiation inactivation technique did not measure the size of Photosystem I polypeptides binding chlorophyll accounting for the small overall target size. The observed functional size came mostly from inactivation of the iron-sulphur centres showing that they are located on separate polypeptides.

Introduction

The initial step in the biological conversion of light energy to chemical energy is a photochemical charge separation followed by electron transfer to create stable products. The photochemical event involves the photooxidation of a special chlorophyll species located in a membrane-bound pigment-protein complex. In higher plants, algae and cyanobacteria there are two types of photochemical reaction centre, Photosystem I (PS I) and Photosystem II (PS II). The reaction centre chlorophylls, P-700 of PS I and P-680 of PS II, are named from their absorption maxima at long wavelengths. Each photosystem also has a distinctive chain of electron acceptors which transfer the electron away from the reaction centre chlorophyll [1]. Electron transfer in PS I from P-700 to ferredoxin involves low-potential membrane-bound iron-sulphur centres which can be detected by EPR at cryogenic temperatures [2,3]. PS I complexes have been partially purified using various non-ionic detergents such as Triton X-100 and the polypeptide and electron carrier components have been studied. The reaction centre chlorophyll P-700 is associated with polypeptides in the 65–70 kDa region of SDS-polyacrylamide gels [4]. In addition several small polypeptides (less than 25 kDa) have been reported as being part of the complex. It has been proposed that some of these

Abbreviations: Mes, 4-morpholineethanesulphonic acid; PS I, Photosystem I; PS II, Photosystem II; Tricine, *N*-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine; SDS, sodium dodecyl sulphate; Chl, chlorophyll.

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polypeptides contain the three iron-sulphur centres called centres A, B and X, which act as electron acceptors. However, no firm polypeptide composition for the PS I reaction centre complex or correlation with known electron carriers has been established [2–14]. Recently, it has been proposed that iron-sulphur centre X could be attached to the polypeptides involved in P-700 binding, perhaps being bound across two polypeptide chains [13,14].

Radiation inactivation has been used to measure the functional molecular size of a variety of enzymes and also the binding or transport activities of proteins. Samples are subjected to doses of high-energy radiation and the residual biological activity measured. Target size analysis involves the calculation of the rate of loss of activity with radiation dose using a simple probability equation. If activity declines as a single exponential function of dose, single hit inactivation of a molecule can be assumed and a linear plot of dose vs. log activity is obtained. The slope of this plot is related to the molecular size either through an empirical equation [15–18] or by calibration with enzyme standards of known molecular size.

Until recently the technique had not been extensively applied to proteins and enzymes containing electron carriers. Many of these proteins are membrane-bound and difficult to purify so that a major advantage of radiation inactivation, the ability to measure the functional size in situ without isolation, can be exploited. The interpretation of results can still be complicated by the presence of detergents which have been shown to affect the rate of inactivation [19,20].

In previous papers we have reported preliminary results from a study of the functional size of water oxidation by PS II and have discussed the use of enzyme standards for molecular size calibration [20–22]. The use of enzyme standards removes the dependence on an accurate measurement of radiation dose and also eliminates factors such as temperature and radiation quenching, which affect the rate of inactivation [20,23].

This paper reports an investigation of the PS I reaction centre using radiation inactivation. Measurements were made on chloroplasts and detergent-purified PS I, utilising both chemical and photochemical reactions. The photochemical activity of PS I was measured at cryogenic temperatures using EPR to demonstrate the photochemical oxidation of P-700 and the concomitant reduction of iron-sulphur centres. At 15–20 K the electron from P-700 is transferred irreversibly through the intermediary carriers A_0, A_1 and centre X (A_2) to either of the two iron-sulphur centres A and B. When centres A and B are lost or chemically reduced, P-700 photo-oxidation becomes reversible as the back reaction between P-700 and centre X proceeds at 15 K. Centres A and B have similar reduction-oxidation (redox) potentials, but distinct EPR spectra [2,3]. In spinach and pea, centre A is the major electron acceptor at 15 K, but an increased proportion of centre B reduction occurs in other species [5]. The use of either glycerol [24], high protein concentration or conditions of high ionic strength lead to the ability to photoreduce a mixture of centres A and B is spinach and pea PS I. Therefore the use of glycerol or bovine serum albumin as cryoprotectants in these studies allowed the effect of irradiation on three components P-700, centre A and centre B to be measured. The results show an unexpectedly small size for reaction centre function indicating that only chemically bound electron carriers such as the iron-sulphur centres were inactivated as a function of protein size. Analysis of the rate of inactivation of the iron-sulphur centres shows that centres A and B are located on different polypeptides with the electron carriers from P-700 to centre X located on a separate polypeptide complex.

Experimental procedures

Preparation of chloroplasts

Chloroplast membranes were prepared from the leaves of market spinach (*Spinacia oleracea*) or greenhouse-grown peas (*Pisum sativum* var Feltham First) by the method described in Ref. 25. All procedures were carried out at 4°C avoiding exposure to bright light. This preparation is referred to as chloroplast PS I and was suspended in 20 mM Mes, 15 mM NaCl, 5 mM $MgCl_2$ and 20% (w/v) glycerol (pH 6.3) (buffer A). Glycerol was omitted from samples to be freeze-dried and replaced by bovine serum albumin protein as specified in the text.

Preparation of purified PS I

Purified PS I was prepared by a method based on that in Ref. 8 using the non-ionic detergent Triton X-100. 20% (w/v) Triton X-100 was added to chloroplast membranes prepared as above to give a concentration of 2 mg chlorophyll (Chl)/ml and 5% Triton X-100. This was incubated at 4°C for 1 h and then centrifuged at $35000 \times g$ for 30 min. The supernatant containing the solubilised PS I was then purified as described in Ref. 8 using hydroyapatite column chromatography. The PS I eluted from this column with 10 mM PO_4, 200 mM NaCl and 0.1% Triton X-100 (pH 6.8) was washed and concentrated by Amicon Diaflo ultrafiltration using a YM100 membrane. It was finally suspended in 100 mM Tricene, 200 mM NaCl and 0.1% Triton X-100 (pH 8.2) for storage either frozen or freeze-dried. The ratio of P-700 to chlorophyll was 1:25 and 1:45 for the preparations used in this study.

Sample preparation and treatment

Either frozen or freeze-dried samples were prepared in order to minimise the secondary reactions of radiation as discussed in Ref. 21.

(a) Frozen samples. 0.3 ml samples containing enzyme standards were flushed with nitrogen gas for 30 min and placed in 0.3 cm diameter silica tubes. Samples were then frozen, stored in liquid nitrogen and transported either in liquid nitrogen or solid carbon dioxide pellets.

(b) Freeze-dried samples. 1 ml samples containing enzymes as specified were frozen in 5 ml graduated Pyrex tubes and freeze-dried overnight avoiding exposure to bright light. The tubes were then flushed with nitrogen gas, sealed with a glass stopper and stored at 4°C until irradiated.

Irradiation procedure

Irradiation of frozen samples was carried out at 77 K in liquid nitrogen using a 16 meV electron beam (MEL SL 75/20 linear accelerator Addenbrookes Hospital, Cambridge, U.K.) as in Ref. 21.

Freeze-dried samples were irradiated under vacuum at room temperature (295–305 K) as in Ref. 23. Tubes were cooled during irradiation using a fan to drive air over solid carbon dioxide and then through holes in the lead block holding samples.

The dose rate was 2 Mrad/min. Freeze-dried samples were irradiated in 2 min treatments followed by a cooling period to prevent overheating of the sample. The radiation dose was checked using Perspex (poly(methylmethacrylate)) dosimetry. Following treatment, all tubes were flushed with nitrogen before thawing or rehydration in order to remove oxygen and traces of ozone [20].

Assays

Chlorophyll *a* and *b* were measured by the method of Arnon using 80% (v/v) acetone [26]. Enzymes were assayed at either 20° or 25°C using duplicate or triplicate assays of each sample. Temperature variation was ±0.5°C and could be corrected for by using control samples. Enzyme assays were as described in Ref. 20 using a Cary 219 spectrophotometer and 1 ml 1 cm path-length quartz cuvettes. Malate dehydrogenase (porcine heart cytoplasmic EC 1.1.1.37) was assayed as the rate of oxidation of NADH monitored at 340 nm using freshly made oxaloacetate solution as substrate. Glucose-6-phosphate dehydrogenase from *Leuconostoc mesenteroides* (E.C. 1.1.1.49) was assayed as the rate of reduction of NAD^+, monitored at 340 nm using glucose 6-phosphate as substrate. Cytochrome *c* (type-III horse heart) was measured as the height of the 550 nm absorption peak in a reduced-minus-oxidised spectrum using automatic baseline correction. P-700 was assayed chemically as the height of the reduced-minus-oxidised optical spectrum at 700 nm. Enzymes and substrates were purchased from Sigma chemical company and reagents were Analar grade.

EPR spectrometry

EPR spectrometry was performed at cryogenic temperatures using a Jeol FE1 X-band spectrometer with 100 kHz field modulation and an Oxford instruments liquid helium cryostat. 0.3 ml samples in 3 mm diameter calibrated quartz tubes were used. Chlorophyll concentration of samples and EPR conditions are described in the text. Samples were prepared as follows. (a) For PS I photochemistry, 10 mM sodium ascorbate was added to samples which were then dark adapted for 15–20 min before freezing in liquid nitrogen in the dark. This reduces P-700 but not PS I electron acceptors. Samples were examined by EPR at

15–20 K and illuminated for 1 min at 15 K with saturating light to induce charge separation in PS I. (b) For full reduction of all three iron-sulphur centres in PS I, samples were flushed with oxygen free nitrogen and sodium dithionite added to 0.2% (w/v). After incubation for 10 min, samples were illuminated for 1 min and then frozen under illumination. The measurement of the size of EPR signals is described in the text.

Data analysis

A Minitab statistical programme (Penn State University, PA) was used for linear regression and analysis of variance. Enzyme activities were expressed as a percentage of control values and target analysis performed as in Ref. 21. Regression lines were checked for error by a plot of standard residuals vs. dose. Molecular mass was estimated by comparison with the protein standards [20]. Errors expressed as standard deviations were approx. 10%. A Tectronix 4051 microcomputer was used to store EPR spectra.

Results and Discussion

Increasing doses of radiation decreased the photochemical activity of detergent purified PS I as shown in Fig. 1. The contributions of P-700, centre A and centre B to the spectrum are shown. As expected the rate of inactivation was several-fold slower in samples irradiated frozen at 77 K than samples irradiated at room temperature. However, using molecular size standards (malate dehydrogenase, glucose-6-phosphate dehydrogenase and cytochrome *c*) added to the sample to calibrate the rate of inactivation, this temperature effect was corrected giving similar functional size estimates for both types of sample.

Fig. 2 shows semi-logarithmic plots of activity vs. dose, which reveal that the rates of inactivation of PS I photochemistry indicate a mass of less than 40 kDa for the PS I reaction centre. Fig. 2A shows the rate of inactivation of P-700 photo-oxidation at 15 K compared to the loss of bulk Chl *a*. This gives a target size of about 30 kDa for P-700. Similar rates of inactivation were seen for both the chemical and photochemical oxidation of P-700. Radiation inactivation depends on the transfer of ionising energy along the polypeptide

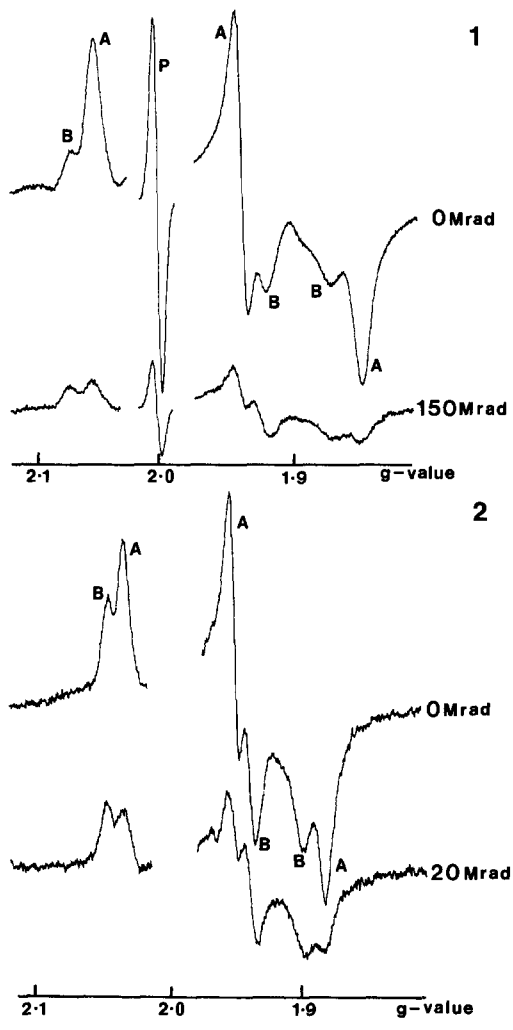

Fig. 1. EPR spectra showing PS I photochemistry at 15 K. Samples were irradiated and EPR samples prepared as given in experimental procedures. (1) Spinach detergent purified PS I irradiated at 77 K, Chl *a* (mg/ml) 0 Mrad 0.75, 150 Mrad 0.63. (2) detergent purified pea PS I irradiated at room temperature, Chl *a* (mg/ml) 0 Mrad 0.72, 20 Mrad 0.58. Spectra shown are the light-induced signals obtained by subtraction of spectra taken before and after 1 min illumination at 15 K. A = centre A, B = centre B, P = P-700. Centres A and B; microwave power, 5 mW; temperature, 15 K; time constant, 0.1 s; scan rate, 12.5 mT/min; modulation amplitude, 1 mT; frequency, 9.05 GHz. P-700 as above, but microwave power 0.1 mW; scan rate, 2.5 mT/min and modulation amplitude, 0.1 mT.

chain to cause damage throughout the protein. Transfer of ionising energy can also sometimes occur between non-covalently bound molecules,

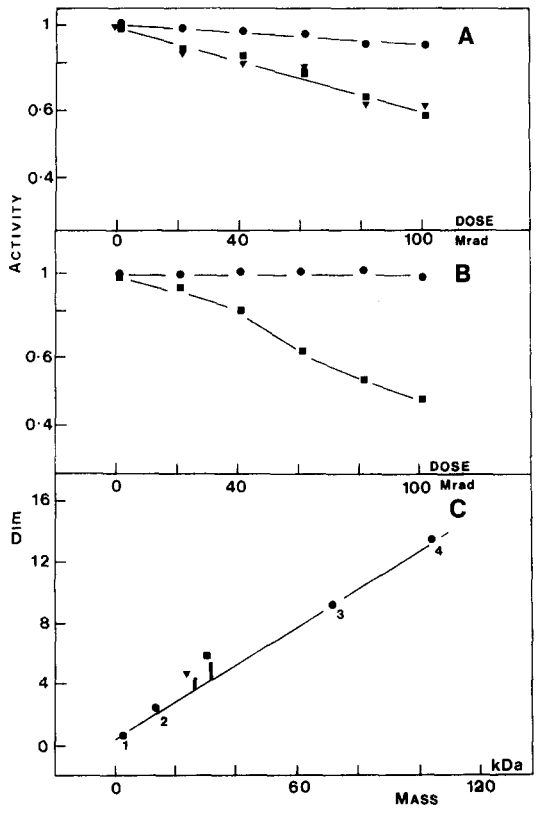

Fig. 2. Rate of inactivation of PS I in frozen samples irradiated at 77 K. Chl concentration of EPR samples, 0.6 mg/ml. The height of the light-induced EPR peak indicated was used as a measure of activity. (A) ●, Chl *a*; ■, *P*-700 oxidised chemically (700 nm peak); ▼, *P*-700 photo-oxidised at 15 K ($g = 2.003$) (B) ●, Photoreduced centre B ($g = 2.065$); ■, photoreduced centre A ($g = 2.05$). (C) Rate of inactivation (E/D, log 'enzyme activity' per radiation dose) vs. molecular mass using mean results from three experiments. ■, P-700/centre A; ▼, P-700/centre B. Standards; 1. Chl *a* (1 kDa); 2. cytochrome *c* (13 kDa); 3. malate dehydrogenase (70 kDa); and 4. glucose-6-phosphate (104 kDa) dehydrogenase. Other conditions, as in experimental procedures and in Fig. 1.

such as the subunits of oligomeric enzymes, e.g., malate dehydrogenase, to produce a single target. However, some oligomeric enzymes behave as monomer targets [20] and energy transfer was also found to be poor between protein and lipid or carbohydrate associated with it [27,28]. The strong evidence associating P-700 with at least one 65–70 kDa polypeptide subunit [4] suggests that an explanation of the consistently small target size (Fig. 2C) is that only a hit at or very close to the reaction-centre chlorophyll or other non-covalently bound chlorophyll molecules will inactivate them. Multiple hits on the reaction-centre core protein would therefore be required to inactivate function [29]. The requirement for multiple hits could also result from multiple subunit involvement in P-700 binding as suggested in Ref. 9 with inactivation requiring hits on several subunits in each reaction centre. Apparently small target sizes have been observed for other polypeptides containing prosthetic groups, rhodopsin [30] and assimilatory NADH-nitrate reductase from *Chlorella* [31] suggesting the possibility of functionally independent domains also occurring in these polypeptides.

Chlorophyll *a* was used as a measure of secondary inactivation processes which includes loss of solubility in samples freeze-dried and rehydrated. The rate of inactivation was found to follow a target size of 5–10 kDa in freeze-dried detergent-purified PS I, but only 2–4 kDa in freeze-dried chloroplast PS I or frozen detergent purified PS I. This clearly shows that the baseline of secondary inactivation is higher in freeze-dried preparations. The ratio of Chl *a* to chlorophyll *b* also fell slowly with increasing radiation dose in all preparations suggesting some shift in absorption peaks as a result of radiation damage. This may make observations based on chemical oxidation of P-700 unreliable when measured at 700 nm.

The light-harvesting chlorophyll-binding proteins have molecular sizes mainly between 25 and 30 kDa, allowing the conclusion that the rate of Chl *a* inactivation is not a function of chlorophyll-binding proteins. The rate of inactivation of bacteriochlorophyll was recently used to investigate the light-harvesting proteins of *Rhodospirillum rubrum* [32]. The authors suggested that the target size of these proteins included both the protein and pigment, allowing the number of pigment molecules on each polypeptide to be estimated. In the present study the results suggest that ionising energy transfer to non-covalently bound chlorophyll is inefficient and does not allow estimation of chlorophyll-binding proteins. The different conclusions of the two studies may result from different radiation conditions or from the fact that the smaller size of the bacterial

binding proteins (6 kDa) does allow single hit inactivation.

Assuming a multihit process of the reaction-centre core protein means that the proteins binding the iron-sulphur centres account for most of the different in the target size seen. In support of this, previous authors have demonstrated that energy transfer is good between protein chains joined by disulphide bridges [33]. It has also been suggested that sulphur atoms within proteins are major sites of radiation damage due to ionisation [18]. Fig. 2B shows the difference between the rates of inactivation of centres A and B in frozen samples. Photoreduced centre B was inactivated more slowly than centre A (Fig. 2B and Fig. 1). Careful checking of EPR conditions ensured that this was not due to changes in microwave power or temperature characteristics of the EPR signals from the centres. Only at radiation doses above 100 Mrad in frozen samples was a significant decrease in photochemical reduction of centre B observed. In all types of sample (freeze-dried chloroplast PS I, detergent PS I and frozen detergent PS I) photoreduction of centre A was inactivated at a higher rate than the rate of loss of irreversibly photooxidized P-700. These results mean that radiation damage causes a net shift in reaction centres from electron transport to centre A to give electron transport to centre B as seen from the spectral changes in Fig. 1. Centre B photoreduction therefore appears almost constant as the creation of centres transferring electrons to centre B matches the rate of loss of such centres through radiation hits. There is therefore no significant transfer of ionising energy between centres A and B or inactivation of the centres would occur in parallel. This suggests that either the iron-sulphur centres are on different polypeptides, isolated domains of the same polypeptide or that nearby radiation hits cause a shift in electron-transport pathway similar to that caused by glycerol or high protein concentrations. The latter two possibilities appear least likely as the changes were always towards increased centre B photoreduction. This would only be expected from random hits if the centre A domain was significantly larger than that of centre B. However, the small target size of each polypeptide (less than 10 kDa) should mean that single hits would destroy all activities on a single polypeptide. It is concluded that centre A is located on a separate polypeptide to centre B and that P-700/centre B forms a smaller target than P-700/centre A. This would result from either the centre A polypeptide being larger than the centre B polypeptide in a branched pathway:

$$\text{P-700} \rightarrow \text{centre X} \rightarrow \begin{matrix}\text{centre A} \rightarrow \\ \text{centre B} \rightarrow\end{matrix}$$

or from polypeptides of similar size in a linear scheme:

$$\text{P-700} \rightarrow \text{centre X} \rightarrow \text{centre B} \rightarrow \text{centre A}$$

Fig. 2C demonstrates the size difference between P-700-centre A and P-700-centre B photochemistry. If it is assumed that this difference reflects the size of the centre A polypeptide, this gives a functional size for centre A of between 5 and 10 kDa in a linear scheme. Many experiments would be required to estimate more accurately the molecular size because of the small target size requring large radiation doses for inactivation. The presence of detergent in purified PS I preparations has also been shown to affect target size measurement [19,20] by increasing size estimates. Therefore measurements on chloroplast PS I would be required to reduce errors further.

Previous experiments to try and resolve the relationship of centre A to centre B used chemical modification [7,9,11] and indicated that centre B is altered prior to centre A. This apparent difference from the results given here may be due to the chemical modification methods depending on access to the iron-sulphur centre in the membrane. Therefore they may be only giving information on the relative position of the centres. The radiation inactivation method does not have any dependence on the position of the centre in the membrane allowing conclusions based on size to be made.

The relationship between centres A and B was also tested by EPR studies using samples which had been reduced and frozen under illumination to give complete reduction of centres A, B and X. Fig. 3 shows spectra of samples with all three iron-sulphur centres reduced. Centres A and B interact when both are reduced in the same reaction centre giving an EPR spectrum as in Fig. 3A.

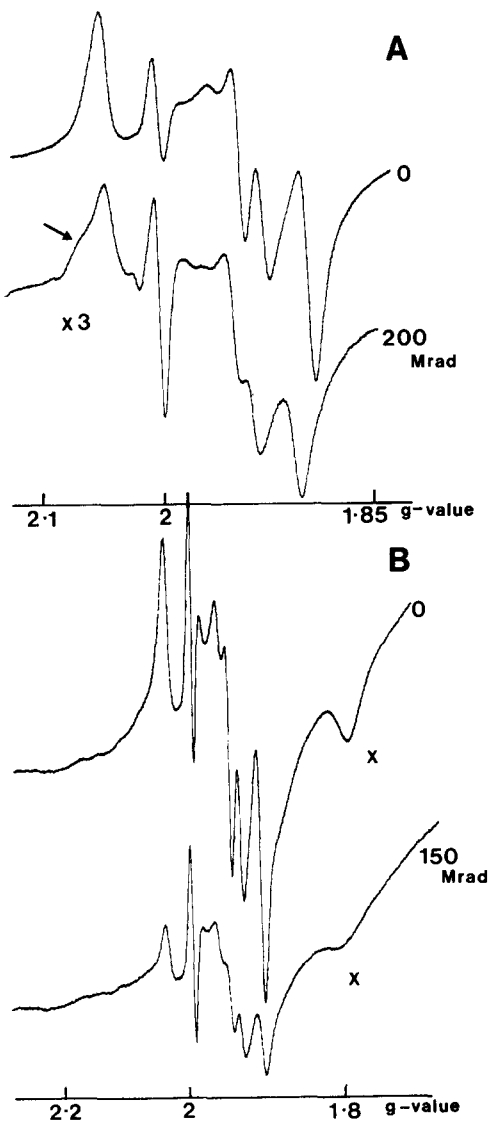

Fig. 3. EPR spectra showing reduced PS I electron acceptors. Samples of purified pea PS I were irradiated dry at room temperature, rehydrated and then reduced by dithionite and frozen under illumination as given in experimental procedures. (A) Spectra at 15 K; (B) spectra at 8.5 K. Other conditions as in Fig. 1. X = g_x resonance of centre X. Note that the spectrum of 200 Mrad in A has been magnified 3-fold.

This interaction requires that the centres are within 1 nm and has led to suggestions that it means that both centres are located on the same polypeptide. The observation of uninteracted centre B in irradiated samples (Fig. 3A arrowed) causing a change in the lineshape of the spectra confirms the different rates of inactivation for centres A and B showing their location on separate polypeptides. The spectrum of uninteracted centre A was not observed, but is more difficult to see as it is hidden by the interaction spectrum.

The spectrum of centre X is observed at 8 K superimposed on that of centres A and B. Fig. 3B shows that the signal for centre X produced by photoreduction was also lost as the radiation dose increased.

Using freeze-dried purified PS I irradiated at room temperature it was possible to achieve doses where significant inactivation of centre B and centre X could be observed (Fig. 4). Fig. 4A gives the rates of inactivation of the photoreduction of centres A and B showing the increased loss of centre B at high doses as centre A is reduced to low levels. In Fig. 4B the rate of inactivation of irreversibly photo-oxidised P-700 at 15 K which was considered above to measure the target size of the P-700$^+$/centre B-couple is compared to that of centre X. The rate of loss of centre X was slower than the rate for P-700/centre B and was similar to the loss of reversibly photo-oxidised P-700 at 15 K in dark-adapted samples which is a rough estimate of centre X photoreduction. Fig. 4C compares the target sizes of the reaction centre when the three iron-sulphur centres are photoreduced by P-700. P-700/centre X appears to be the core of the reaction centre as the rate of loss of centre X photoreduction matched the loss of chemically oxidisable P-700. The small overall target size probably results from a multiple hit requirement as discussed above. Chemical reduction of centres A and B by dithionite at pH 10 could have been used to measure the size of centres A + B using the interacted EPR signal, but this measurement did not produce a linear response between radiation dose and size. It therefore only gave a rough estimate of 15–25 kDa for centre A + B which does not allow distinction between a branched or linear chain. Assuming a linear chain gives a size of 5–10 kDa for both centre A and B as shown by the difference between the rates of inactivation in Fig. 4C. A branched electron-transport chain would mean centre A being about 15 kDa and centre B 5–10 kDa.

The results show some agreement with those of Golbeck and Cornelius [14] who provided evi-

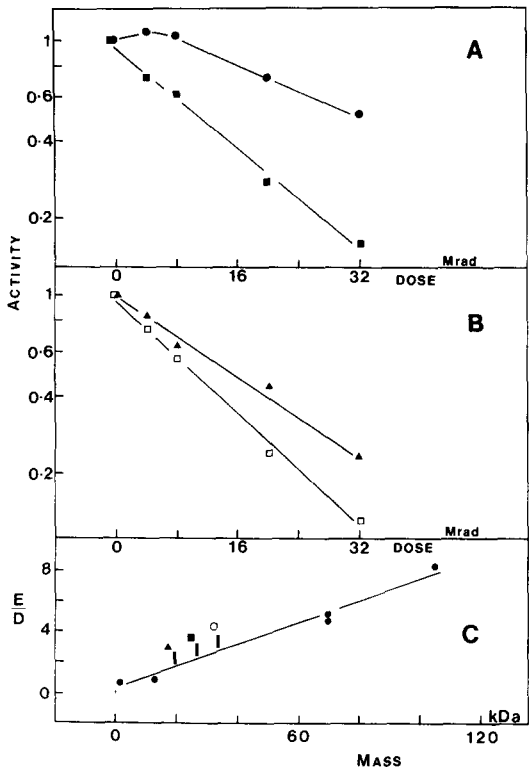

Fig. 4. Radiation inactivation of PS I samples irradiated dry at room temperature. (A) ●, Photoreduced centre B ($g = 2.065$); ■, photoreduced centre A ($g = 2.05$). (B) □, P-700 irreversibly photo-oxidised at 15 K; ▲, photoreduced centre X (g_X). (C) Rate of inactivation vs. molecular mass using the mean rates from three experiments. Target size of: ▲, P-700/centre X; ■, P-700/centre B; ○, P-700/centre A. Standards as shown in Fig. 2.

dence for a close association of centre X with the P-700 containing polypeptide in a core complex. Bonnerjea et al. [7] using chaotropic agents proposed that centres A and B were located on a 19 kDa polypeptide and Moller et al. [6] using radiolabelled iron incorporation identified 15 and 18 kDa polypeptides with the iron-sulphur centres. These results only compare with those given here if there is a branched chain of electron carriers with centre A located on the larger polypeptide. Guikema and Sherman [12] and Lagoutte et al. [10] also using radiolabelling both found evidence for PS I iron-sulphur centres on 8–10 kDa polypeptides although it was suggested that these contain both centres A and B. The radiation inactivation experiments reported here provide positive evidence that centres A and B are located on separate polypeptides.

Redox titration of centres A and B after doses of radiation could provide a definitive molecular size for each centre. However, much larger amounts of irradiated sample would be required than is at present possible. Also difficulties arising from the overlap of the appearance of the centre A and centre B EPR signals in a redox titration may be difficult to interpret as the ratios of the two centres change. Future improvements to the radiation technique could, however, make these experiments possible.

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