PHOTOREDUCTION OF FERREDOXIN BY CHLOROPLASTS WITH OR WITHOUT AN ACCOMPANYING PHOTOREDUCTION OF THE BOUND IRON–SULFUR CENTERS

Contrasting effects of electron donors to photosystems I and II

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1. Introduction

This communication reports new evidence consonant with a recent finding [1] of ferredoxin photoreduction by water when the photoreduction of the bound iron–sulfur centers associated with photosystem I of chloroplasts [2–4] was strongly inhibited.

Chloroplasts photoreduce ferredoxin by electrons that originate from water, via photosystem II, or by electrons supplied directly to photosystem I by artificial donors that bypass photosystem II [5]. Contrary to expectations, only with a direct donor to photosystem I (ascorbate/DCIP) was the photoreduction of ferredoxin by chloroplasts [1], like the photoreduction of ferredoxin by cyanobacterial membrane fragments [6], unequivocally associated with the photoreduction of the bound iron-sulfur centers. With water as donor, ferredoxin was found in [1] to be in a predominantly reduced steady state, even when the photoreduction of the bound iron–sulfur centers was inhibited by DBMIB (2,5-dibromo-3-methyl-6-isopropyl-1,4-benzoquinone). DBMIB is an antagonist of plastoquinone, the chloroplast component deemed essential for electron transfer from photosystem II to photosystem I [7,8].

It appeared, therefore, that, with water as the electron donor, the photoreduction of the bound iron–sulfur centers by photosystem I was an accompaniment but not a prerequisite for the photoreduction of ferredoxin. Normally, electrons originating from water reduced both ferredoxin and the bound iron–sulfur centers but, when electron transport between photosystems II and I was impaired, ferredoxin was photoreduced by water without the involvement of the bound iron–sulfur centers associated with photosystem I [1].

Such a far-reaching interpretation was at variance with the prevailing concept of photosynthetic electron transport [5], which envisions that with either water or a direct donor to photosystem I the reduction of ferredoxin, whose midpoint potential is –420 mV [9,10], is obligatorily linked to the reduction of the bound iron–sulfur centers A and B [11] of photosystem I, whose midpoint potentials are in the region from –530 mV to –580 mV [12,13]. It was deemed desirable, therefore, to test the validity of this unconventional interpretation [1] by other experimental approaches in which the photoreduction of ferredoxin by a donor to photosystem I could be compared with the photoreduction of ferredoxin by a donor to photosystem II.

Reported here are experiments with Tris-treated chloroplasts that lost the ability to use water as donor but which retained the ability to photoreduce ferredoxin (and $NADP^+$) with artificial donors to either photosystem II or I [14]. The aim of the investigation was to determine whether Tris-treated chloroplasts would also show different patterns of association between the reduction of ferredoxin and the reduction of the bound iron–sulfur centers (henceforth referred to as the bound centers), depending on whether a photosystem II or a photosystem I donor was used.

The results show that this proved to be the case.

Abbreviations: DPC, diphenylcarbazide; DCIP, $DCIPH_2$, oxidized and reduced forms of 2,6-dichlorophenolindophenol; DCMU, 3-(3',4'-dichlorophenyl)-1,1-dimethylurea (diuron); EPR, electron paramagnetic resonance

With a photosystem I donor, the photoreduction of ferredoxin was accompanied by the photoreduction of the bound centers. By contrast, the addition of a photosystem II donor to Tris-treated chloroplasts gave only a photoreduction of ferredoxin; there was no evidence for a photoreduction of the bound centers, regardless of whether ferredoxin was included in, or excluded from, the reaction mixture.

2. Methods

Chloroplasts were isolated from spinach leaves (*Spinacia oleracea*, var. Marathon or High Pack) grown in a greenhouse in nutrient solution culture [15] and freshly harvested before each experiment. The preparation used consisted of osmotically disrupted (broken) chloroplasts capable of complete electron transport from water to $NADP^+$ and of ferredoxin-catalyzed cyclic photophosphorylation [16]. The chloroplasts were used directly or after Tris treatment as in [14] except that the incubation time with 0.8 M Tris–HCl was extended to 1 h. Chlorophyll was estimated [15], ferredoxin was isolated and purified [17] (by R. K. Chain), and the photoreduction of $NADP^+$ was measured [18] as described. Glucose oxidase (type VII), bovine catalase, and $NADP^+$ were purchased from Sigma Chemical Co. (St Louis, MO) and diphenyl carbazide (DPC) was from Eastman Kodak Co. (Rochester, NY).

The photorreduction of ferredoxin and of the membrane-bound iron–sulfur centers was measured by electron paramagnetic resonance (EPR) spectroscopy. The chloroplasts (in their respective reaction mixtures) were placed in quartz EPR tubes (3 mm internal diameter) pre-gassed with nitrogen. The tubes were illuminated first at a physiological temperature (293 K) for 30 s then, with illumination continued, immersed for 30 s in liquid nitrogen, contained in a silvered dewar with a window that admitted light during freezing. Monochromatic illumination (664 nm) was provided by a light beam from a quartzline lamp (type DXN, 1000 W). The light beam was passed through heat-absorbing and interference filters (Baird-Atomic Co., Medford, MA).

First-derivative EPR spectra of the frozen samples were obtained with a Bruker Instruments Co. (Billerica, MA) X-band spectrometer (model ER200tt) (equipped with a 20 cm (8 in.) magnet) operated at a frequency of 9.18 GHz and were recorded after processing by a digital signal averager (model 1070, Nicolet Instru. Corp., Madison, WI). The frozen samples in the quartz EPR tubes were further cooled with liquid helium to either 20 K or 60 K by an Oxford Instruments temperature controller (model DTC) and cryostat (model ESR9) equipped with a quartz dewar cell (made by J. Scanlon, Solvang, CA).

3. Results

As stated in section 2, the Tris treatment of chloroplasts was longer than usual [14] in order to minimize any remaining capacity of chloroplasts to photooxidize water. Fig.1 shows that the Tris-treated chloroplasts were no longer able to photoreduce $NADP^+$ with water as donor (left trace). They were able, however, to photoreduce $NADP^+$ when supplied with either a photosystem II donor, diphenyl carbazide (DPC) [19], in the absence of diuron, or a photosystem I donor, ascorbate/DCIP [20], in the presence of diuron. With the photosystem II donor, the photoreduction of $NADP^+$ was inhibited by diuron (fig.1).

In the EPR traces presented below, the extent of ferredoxin reduction is indicated by the amplitude of characteristic EPR signals at $g = 1.89, 1.96$ (main

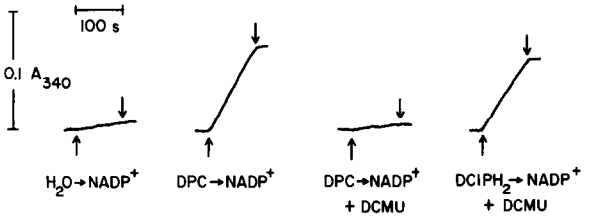

Fig.1. Photoreduction of $NADP^+$ by Tris-treated chloroplasts supplied with different electron donors. Reaction mixtures contained Tris-treated chloroplasts (equiv. 50 μg chl/ml), a saturating amount of ferredoxin–$NADP^+$ reductase, 0.01 mM spinach ferredoxin, 50 mM tricine buffer (pH 8.2), 10 mM $MgCl_2$ and 2 mM $NADP^+$. In addition, the following were added where indicated: 0.5 mM DPC (diphenylcarbazide), 0.01 mM DCMU (diuron) and, for the DCIPH_2 system, 10 mM Na-ascorbate and 0.1 mM DCIP. The reaction mixtures were illuminated at room temperature in cuvettes (2 mm lightpath) open to air. Arrow up, light on; arrow down, light off. Monochromatic illumination: 664 nm, 1×10^4 ergs . cm^{-2} . s^{-1}. The rates (μmol NADPH . mg chl^{-1} . h^{-1}) of light-induced electron transport were, from left to right: 2,35,1,35. In the untreated chloroplasts, the rates (not shown) for $H_2O \rightarrow NADP^+$ and DCIP$H_2 \rightarrow NADP^+$ were 108 and 37, respectively.

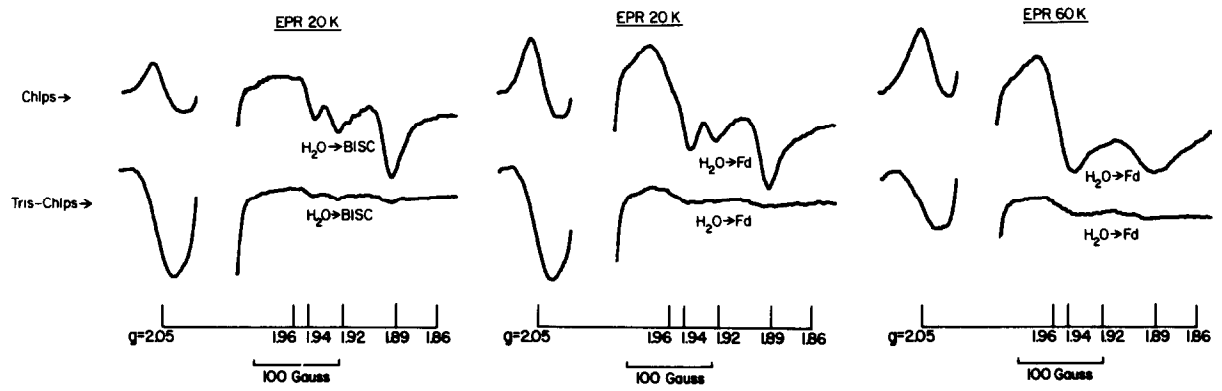

Fig.2. Effectiveness of Tris-treatment of chloroplasts in abolishing the photoreduction of ferredoxin (Fd) and the bound iron–sulfur centers (BISC) with water as electron donor. The extent of ferredoxin reduction is indicated by the amplitude of the EPR signals at g = 1.89, 1.96 and 2.05 and of the bound iron–sulfur centers at g = 1.89, 1.92, 1.94 and 2.05. The reaction mixture, equilibrated with N_2, contained 50 mM Tricine buffer (pH 8.2), 10 mM $MgCl_2$, 2.5 mM ADP, 2.5 mM K_2HPO_4, 10 mM glucose, 3% methanol and (per ml), 7500 units catalase, 13 units glucose oxidase, and untreated or Tris-treated chloroplasts 1 mg chl equiv. Where indicated, 0.01 mM spinach ferredoxin was added. Monochromatic illumination, 664 nm, 1×10^5 ergs . cm^{-2} . s^{-1}. EPR spectra were recorded at 20 K and 70 K. Spectrometer field setting, 3450 ± 200 G; microwave power, 10 mW; modulation amplitude, 10 G. Gain 1×10^5.

signal) and 2.05. The reduced bound centers give signals at g = 1.86, 1.94 and 2.05 (center A) and at g = 1.89, 1.92 and 2.05 (center B). In fully reduced chloroplast preparations, the g = 1.86 signal of center A seems to undergo a g-value shift to 1.89, although the other g-values of center A remain unchanged [2–4,12,13,21]. Because of the considerable overlap between the signals of the reduced ferredoxin and the reduced bound centers, the EPR tubes were scanned at 20 K and 60 K. The scan at 20 K gave signals of both reduced ferredoxin and the reduced bound centers but at 60 K the EPR signals of the bound centers broadened and ceased to be detectable [6]. Thus, the EPR scan at 60 K served as a measure of reduced ferredoxin only.

The upper traces in fig.2 show that the untreated chloroplasts, using water as donor, photoreduced the bound centers alone in the absence of ferredoxin (left) and reduced both ferredoxin and the bound centers when ferredoxin was present (middle and right traces). The lower traces in fig.2 shows that the Tris treatment completely abolished the capacity of untreated chloroplasts to use water for the reduction of the bound centers and of ferredoxin.

The addition of the photosystem II donor (DPC) restored the ability of the Tris-treated chloroplasts

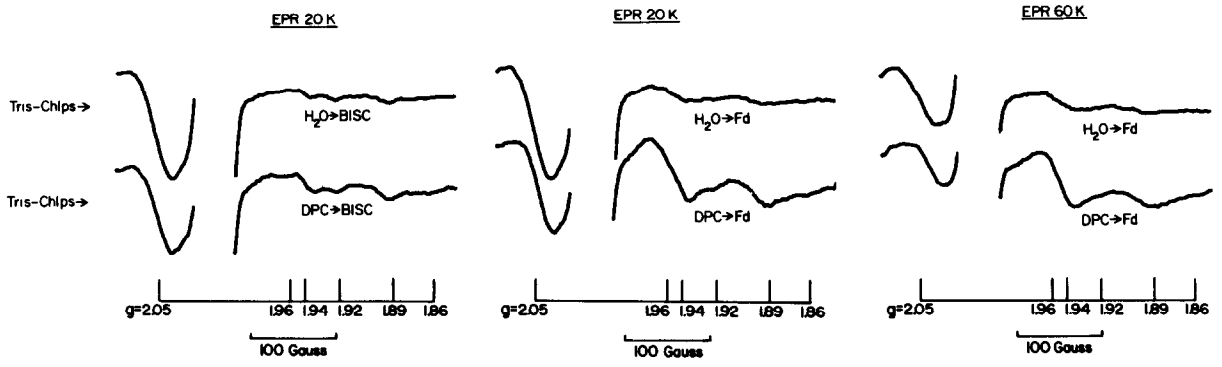

Fig.3. Photoreduction of ferredoxin (Fd) and bound iron–sulfur centers (BISC) by Tris-treated chloroplasts with a photosystem II donor (DPC). Experimental conditions and EPR settings were as in fig.2 except that, where indicated, 0.5 mM DPC was added.

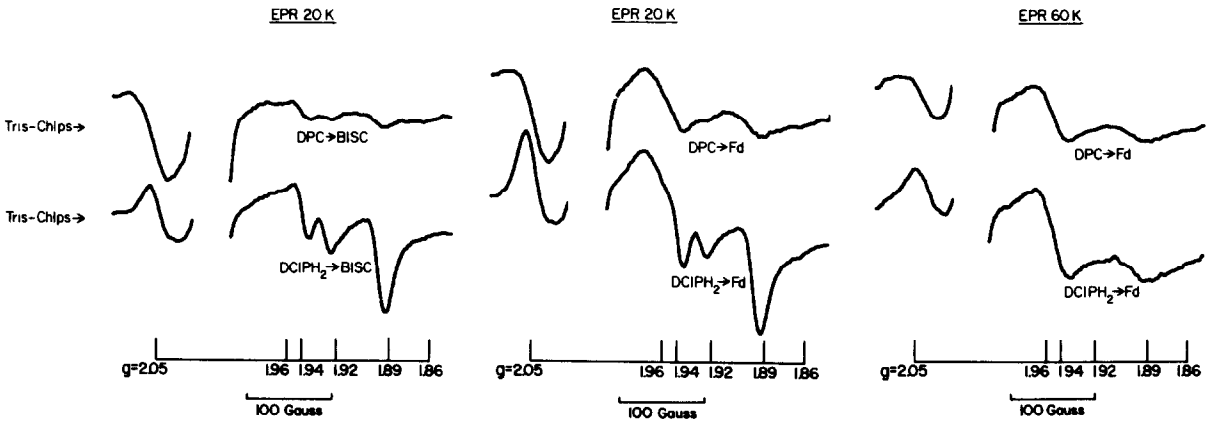

Fig.4. Comparison of the photoreduction of ferredoxin (Fd) and bound iron–sulfur centers (BISC) by Tris-treated chloroplasts supplied with either a photosystem II donor (0.5 mM DPC) or a photosystem I donor ($DCIPH_2$) (10 mM Na-ascorbate/0.1 mM DCIP + 0.01 mM diuron). Other experimental conditions and EPR settings were as in fig.2.

to photoreduce ferredoxin (fig.3, middle and right traces) but not the ability to photoreduce the bound centers (fig.3, left). The contrasting effects of the photosystem I donor ($DCIPH_2$) are shown in fig.4. Chloroplasts supplied with $DCIPH_2$ photoreduced the bound centers alone when ferredoxin was not included in the reaction mixture (fig.4, lower left trace) and photoreduced both ferredoxin and the bound centers when ferredoxin was present (fig.4, lower middle and right traces).

With the photosystem I donor ($DCIPH_2$) the photoreduction of ferredoxin and the bound centers was measured in the presence of diuron (see legend to fig.4) whereas with the photosystem II donor (DPC) the photoreduction of ferredoxin was, like the photoreduction of $NADP^+$ shown in fig.1, completely inhibited by diuron (fig.5).

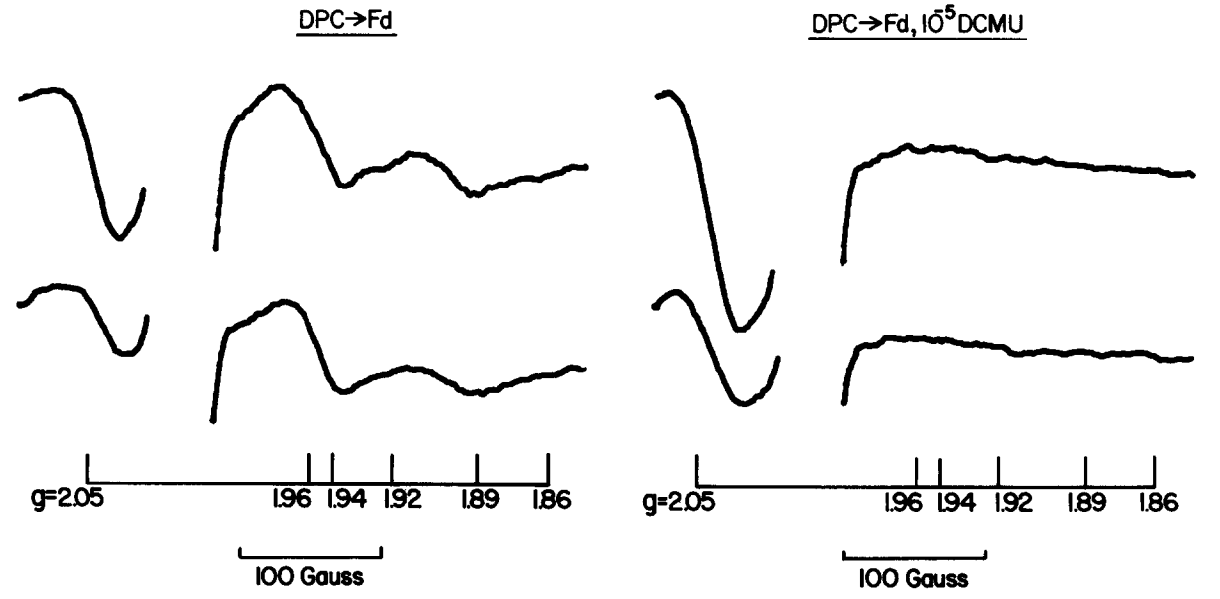

Fig.5. Inhibition by diuron (DCMU) of photoreduction of ferredoxin with DPC. Upper EPR traces were recorded at 20 K, lower at 60 K. Experimental conditions as in fig.2 except that, where indicated, 0.5 mM DPC and 0.01 mM DCMU were added. EPR settings were also as in fig.2 but the photographic enlargement was greater.

4. Discussion

Since its introduction [14], the Tris treatment of chloroplasts has been widely used to inhibit the photooxidation of water by photosystem II, especially in situations in which it was desired to have a mild and reversible [22] treatment that would not otherwise impair the integrity of photosystem II or affect that of photosystem I. In the present investigation, Tris-treated chloroplasts exhibited two different, donor-dependent patterns of ferredoxin reduction. With a photosystem I donor, the steady-state photoreduction of ferredoxin was accompanied by the steady-state photoreduction of the bound iron-sulfur centers. By contrast, with a photosystem II donor, there was a diuron-sensitive, steady-state photoreduction of ferredoxin with no evidence for a concomitant photoreduction of the bound centers (fig.4, center traces; fig.5).

Also to be noted are the contrasting effects of the two donors on the photoreduction of the bound centers alone. Without added ferredoxin, the bound centers were photoreduced with the photosystem I donor but not with the photosystem II donor (fig.4, left traces). This difference argues against the possibility that the observed inability of the photosystem II donor to photoreduce the bound centers when ferredoxin was present resulted from ferredoxin acting as a terminal trap for electrons flowing through the bound centers. The data provide no evidence for a significant electron flow in the Tris-treated chloroplasts from the photosystem II donor to the bound centers. In marked contrast is the unequivocal evidence in the presence or absence of ferredoxin, for the photoreduction of the bound centers by the photosystem I donor in Tris-treated chloroplasts (fig.4), untreated chloroplasts [1], and cyanobacterial membrane fragments [6].

The bound centers and ferredoxin were fully photoreduced by water in the untreated chloroplasts (fig.2, upper traces) and by $DCIPH_2$ in the Tris-treated chloroplasts (fig.4, lower traces), as evidenced by similar EPR signal intensities obtained when excess dithionite (plus illumination) was used for the reduction of equal concentrations of chloroplasts and/or ferredoxin (not shown). Thus, our results are not based on a small fraction of the total possible change in either the bound centers or ferredoxin. Moreover, in the EPR experiments, the relative proportions of these components were not too dissimilar from those in intact spinach chloroplasts. In the EPR experiments the molar ratio ferredoxin:chlorophyll was 1:100 and that of centers (A+B):chlorophyll is estimated to be 1:200. In intact spinach chloroplasts the ferredoxin:chlorophyll ratio is about 1:400 [9] and the ratio of centers (A+B):chlorophyll is about 1:200 (based on ratios of P700:chlorophyll = 1:400 [23]; center A:chlorophyll = 1:400 [23] and center A:center B = 1:1 [24]).

It is possible that for reasons not yet apparent there was a reoxidation of the bound centers when DPC was the donor and not when $DCIPH_2$ was the donor. Such a possibility would allow for an interpretation of our data within the bounds of the conventional Z scheme. Unless such evidence is forthcoming consideration should be given to an alternative hypothesis of electron transport in photosynthesis. These results and those in [1] are consistent with an alternative hypothesis, to be described more fully separately, that there are two mechanisms for the photoreduction of ferredoxin by chloroplasts, one involving primarily photosystem I and linked to the role of ferredoxin as a catalyst of cyclic photophosphorylation [16,25] and another mechanism involving primarily photosystem II and linked to the role of ferredoxin as the key electron carrier in the photoreduction of $NADP^+$ by water [26]. Only in the first mechanism, involving photosystem I, is the photoreduction of ferredoxin envisioned to be in series with the photoreduction of the bound iron-sulfur centers.

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