Journal of Bioenergetics (1976) 8, 83–92

Evidence for Multiple Sites of Ferricyanide Reduction in Chloroplasts*

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Revise received 3 July 1975

Abstract

Various sites of ferricyanide reduction were studied in spinach chloroplasts. It was found that in the presence of dibromothymoquinone a fraction of ferricyanide reduction was dibromothymoquinone sensitive, implying that ferricyanide can be reduced by photosystem I as well as photosystem II. To separate ferricyanide reduction sites in photosystem II, orthophenanthroline and dichlorophenyl dimethylurea inhibitions were compared at various pH's. It was noted that at low pH ferricyanide reduction was not completely inhibited by orthophenanthroline. At high pH's, however, inhibition of ferricyanide reduction by orthophenanthroline was complete. It was found that varying concentration of orthophenanthroline at a constant pH showed different degrees of inhibition. In the study of ferricyanide reduction by photosystem II various treatments affecting plastocyanin were performed. It was found that Tween-20 or KCN treatments which inactivated plastocyanin did not completely inactivate ferricyanide reduction. These data support the conclusion that ferricyanide accepts electrons both before and after plastoquinone in photosystem II.

Introduction

Ferricyanide is thought to accept electrons at several sites in the chloroplast electron transport chain. Trebst et al. [1] found that

* Supported by NSF Grant BMS 74-19689.

† Abbreviations used: DCMU = 3-(3,4-dichlorophenyl)-1,1-dimethyurea; MV = methyl viologen; DBMIB = 2,5-dibromothymoquinone; DMBQ = 2,6-dimethyl benzoquinone; OP = 1,10-orthophenanthroline; TMPD = tetramethyl-*p*-phenylenediamine; PS I = photosystem I; PS II = photosystem II; SN = sucrose–sodium chloride chloroplasts.

ferricyanide accepts electrons in photosystem II in sonicated and detergent-treated chloroplasts. However, in whole chloroplasts ferricyanide reduction occurs in photosystem I. Recently Horton and Cramer [2] have reported that cytochrome *f* is accessible to ferricyanide.

While studying ferricyanide reduction in chloroplasts it was noted that only a fraction (30%–50%) of the ferricyanide reduction rate was active in the presence of dibromothymoquinone, indicating that multiple sites might be involved. We then tried to ascertain how many sites of ferricyanide reduction are present in photosystem II. This aim was attained by observing the effects over a range of pH's and various concentrations of orthophenanthroline inhibition in PS II and by comparing ferricyanide reduction rates in Ort and Izawa's [3] whole chloroplasts with those of sucrose–sodium chloride chloroplasts (SN).

Methods and Materials

Spinach leaves were acquired from the local market. Chloroplasts were prepared using Jagendorf and Avron's method [4] for sucrose–sodium chloride (SN) chloroplasts and Ort and Izawa's [3] method for whole chloroplasts. Most photosystem I and photosystem II assays were performed as reported previously [5]. Oxygen evolution in presence of ferricyanide was measured polarographically with a Clarke-type electrode. The $H_2O \rightarrow$cobaltinitrite reaction was done according to Barr, Rosen, and Crane [6].

Photosystem II reactions were run with phosphate buffer at pH 7.0 and photosystem I reactions at pH 8.0 except in the pH series, where pH's from 5.5 to 8.5 were tested. 0.04 μg DBMIB was added to stop electron flow beyond plastoquinone *A* when the reduction of ferricyanide was measured between the DCMU inhibition site and PQA in photosystem II.

Orthophenanthroline and DBMIB were added to chloroplasts in ethanol not to exceed 20 μl ethanol/1.5 ml.

Tween washes of chloroplasts were done by briefly stirring chloroplasts containing 1.0 mg chlorophyll in 50 ml 1% Tween-20 solution [6]. A pellet of Tween-washed chloroplasts was collected by centrifuging the suspension at 1,200 × g for 10 min. This pellet was resuspended in 0.8 ml SN (0.4 *M* sucrose with 0.05 *M* NaCl) for use in assays.

KCN treatment of chloroplasts to inactivate plastocyanin was performed according to Izawa et al. [7].

Results

While studying the effect of various concentrations of orthophenanthroline on ferricyanide reduction in SN chloroplasts (Fig. 1), we

decided to see if the degree of inhibition varied with pH. It was found (Fig. 2) that ferricyanide reduction in presence of DBMIB by photosystem II had two pH optima, at pH 6.0 and 7.5, while inhibition by orthophenanthroline showed a different pattern. At pH 6.0, 5 μg OP gave about 10% inhibition while at pH 7.5 inhibition was complete in SN chloroplasts. In Ort and Izawa's [3] whole chloroplasts (Figs 3 and 4) higher concentrations of OP were necessary for complete inhibition at pH 7.5. Five micrograms of OP at pH 6.0 showed about 50% inhibition. Other factors influencing ferricyanide reduction are listed in Table I. These include ethanol, polylysine, and other chelators, such as bathophenanthroline sulfonate.

The differential effects of OP at pH 6.0 and 7.5 cannot be explained by assuming that at low pH OP does not penetrate to reach its active site, as can be seen from testing OP inhibition of other chloroplast reactions (Table II). In this table the effects of OP inhibition at pH 5.5 and 8.0 are compared in such PS II reactions as $H_2O \rightarrow$ indophenol and $H_2O \rightarrow$ dimethylbenzoquinone against the $H_2O \rightarrow$ ferricyanide reaction. From the rates obtained in the presence of OP it can be seen that at pH 5.5 OP inhibition is strongest in the $H_2O \rightarrow$ dimethylbenzoquinone pathway (~50%). $H_2O \rightarrow$ methyl viologen and $H_2O \rightarrow$ cobaltinitrite [6] reactions that involve both photosystems also show differential effects of OP

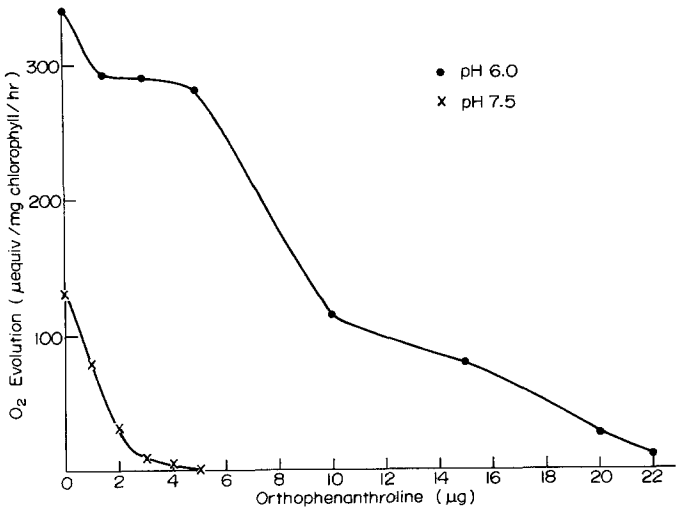

Figure 1. The effects of increasing orthophenanthroline concentration on ferricyanide reduction in photosystem II of sucrose–sodium chloride (SN) spinach chloroplasts. The reaction mixture contained in 1.5 ml total volume: chloroplasts with 0.05 mg chlorophyll, 20 m moles phosphate buffer at pH 6.0 or 7.5, 2.5 m moles ferricyanide, 7.5 m moles NH_4Cl, 0.2 μmole DBMIB and various concentrations of OP.

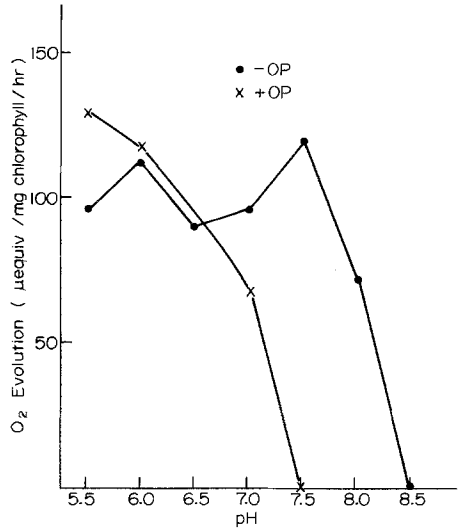

Figure 2. pH effects on orthophenanthroline inhibition of ferricyanide reduction in photosystem II of sucrose-sodium chloride (SN) spinach chloroplasts. Reaction mixture as in Fig. 1 except with varying pH's.

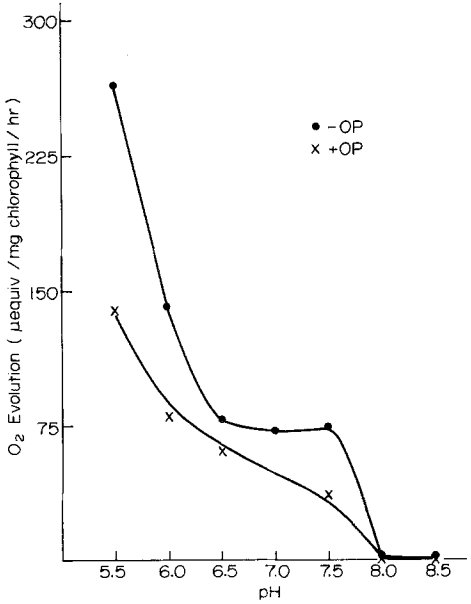

Figure 3. pH effects on orthophenanthroline inhibition of ferricyanide reduction in photosystem II of Ort and Izawa's spinach chloroplasts. Reaction mixture as in Fig. 1, pH varied from 5.5 to 8.5.

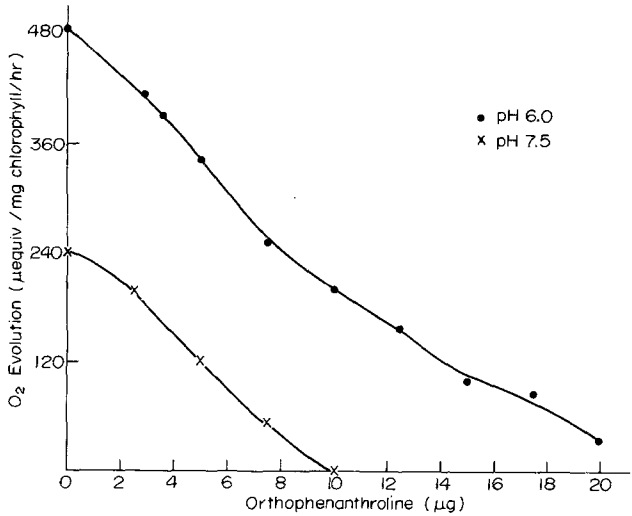

Figure 4. The effects of increasing orthophenanthroline concentrations on ferricyanide reduction in photosystem II of Ort· and Izawa's spinach chloroplasts. The reaction mixture as in Fig. 1.

TABLE I. Factors affecting ferricyanide reduction in spinach chloroplasts[a]

Reaction	Additions	Rate (μequiv/mg chlorophyll/h)
$H_2O \rightarrow K_3Fe(CN)_6$	None	310
	DCMU (3 μg/ml)	0
	DBMIB (0.04 μg/ml)	113
	Polylysine (0.2 mg)[b]	
	DBMIB + PL (0.2 mg)	152
	Bathophenanthroline sulfonate (0.25 mg)	310
$H_2O \rightarrow K_3Fe(CN)_6$ (+DBMIB)	Ethanol (25λ)	79
	Ethanol (50λ)	28
	Ethanol (100λ)	45
$H_2O \rightarrow K_3Fe(CN)_6$		292
	Ethanol (25λ)	169
	Ethanol (50λ)	113
	Ethanol (100λ)	90
$H_2O \rightarrow K_3Fe(CN_6)$(+DBMIB)		96
$H_2O \rightarrow K_3Fe(CN_6)$(+DBMIB) preilluminated 2 min		96

[a] The reaction mixture contained in 1.5 ml total volume: chloroplasts (50 μg chlorophyll), 37.5 m moles Trizma-Mes, pH 7.0, 3 m moles NH_4Cl, 3 m moles $MgCl_2$, 2.5 m moles $K_3Fe(CN)_6$, and 0.06 μg DBMIB where indicated.
[b] 30,000 M.W.

TABLE II. Inhibition of various photosynthetic partial reactions by orthophenanthroline at different pH's[a]

Reaction	Additions	pH	Rate (μequiv/mg chlorophyll/h)	Inhibition (%)
$H_2O\rightarrow$ ferricyanide +DBMIB	None	5.5	316	—
	Orthophenanthroline (5μg)	5.5	237	25
	None	8.0	68	—
	Orthophenanthroline (5μg)	8.0	0	100
$H_2O\rightarrow$ indophenol +DBMIB	None	5.5	265	—
	Orthophenanthroline (5μg)	5.5	186	30
	None	8.0	45	
	Orthophenanthroline (5μg)	8.0	0	100
$H_2O\rightarrow$ DMBQ +DBMIB	None	5.5	784	—
	Orthophenanthroline (5μg)	5.5	338	56
	None	8.0	259	—
	Orthophenanthroline (5μg)	8.0	0	100
$H_2O\rightarrow$ methyl viologen	None	5.5	406	—
	Orthophenanthroline (5μg)	5.5	350	14
	None	8.0	530	—
	Orthophenanthroline (5μg)	8.0	305	43
$H_2O\rightarrow$ cobaltinitrite	None	5.5	203	—
	Orthophenanthroline (5μg)	5.5	124	39
	None	8.0	214	—
	Orthophenanthroline (5μg)	8.0	158	26

[a] The reaction mixture for the $H_2O\rightarrow$ferricyanide reaction was as in Table I, the $H_2O\rightarrow$indophenol reaction contained 2.5 m moles indophenol in place of ferricyanide, the $H_2O\rightarrow$DMBQ reaction contained 2.5 m moles DMBQ in place of ferricyanide, the $H_2O\rightarrow$MV reaction contained 5 m moles MV in place of ferricyanide, and the $H_2O\rightarrow$cobaltinitrite reaction contained 275 μg cobaltinitrite in place of ferricyanide.

inhibition at the two pH extremes but they are the opposite of those observed with ferricyanide: less inhibition (43%) at pH 7.0 with $H_2O\rightarrow$methyl viologen and more inhibition (39%) with $H_2O\rightarrow$cobaltinitrite.

A study of the effect of storage on OP inhibition patterns at low and high pH in SN chloroplasts (Fig. 5) showed that ferricyanide reduction rates assayed at pH 7.5 decreased faster than at pH 6.0 and that OP inhibition was always stronger at higher pH.

Ferricyanide accepts electrons in photosystem I in whole chloroplasts and in broken chloroplasts without DBMIB, but its exact site of action is unknown. In this study ferricyanide reduction by PS II after the DBMIB inhibition site was compared in normal and in Tween- or KCN-washed chloroplasts (Table III). After Tween-20 washes, the ascorbate + TMPD

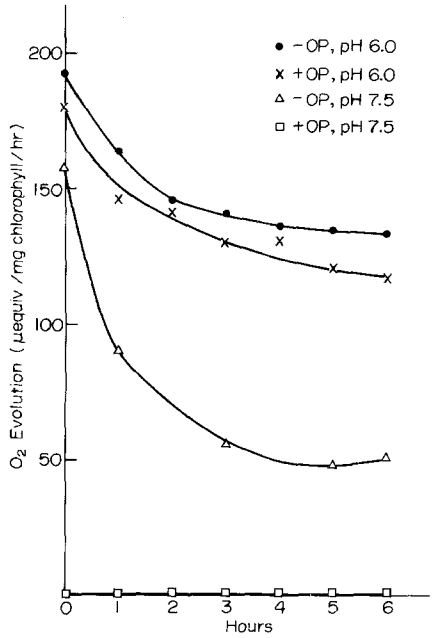

Figure 5. A time-course study of orthophenanthroline inhibition of ferricyanide reduction by photosystem II at pH 6.0 and 7.5 in sucrose–sodium chloride (SN) chloroplasts. Reaction mixture as in Fig. 1.

→methyl viologen and the H_2O→methyl viologen reaction were completely inactivated by these treatments, which remove plastocyanin, but ferricyanide reduction rates decreased only slightly. Conversely, the H_2O→methyl viologen and the ascorbate + TMPD→methyl viologen reactions could be restored by exogenous plastocyanin, but ferricyanide reduction is not increased. KCN wash, which also inactivated the ascorbate + TMPD→methyl viologen pathway through the removal of copper from plastocyanin, did not significantly inactivate ferricyanide reduction by PS II in SN chloroplasts.

Discussion

It is known that electrons from the electron transfer chain can be accepted by ferricyanide at various sites depending on the structural configuration of chloroplasts [8]. In sonicated and detergent-treated chloroplasts ferricyanide accepts electrons in photosystem I unless electron flow through PS I is prevented by an inhibitor [1]. Horton and

TABLE III. DBMIB-sensitive ferricyanide reduction in photosystem II of spinach chloroplasts[a]

Reaction	Treatment	Additions	Rate[b]
$H_2O \rightarrow MV$	Water wash	None	283
	Tween wash	None	0
	Tween wash	PC[c] (0.13 mg)	254
Asc. + TMPD→MV	Water wash	None	1514
	Tween wash	None	68
	Tween wash	PC (0.13 mg)	1416
$H_2O \rightarrow K_3Fe(CN)_6$	Water wash	None	433
	Tween wash	None	335
	Tween wash	PC (0.04 mg)	455
	Tween wash	PC (0.13 mg)	303
Asc. + TMPD→MV	KOH wash	None	1242
	KCN wash	None	282
$H_2O \rightarrow K_3Fe(CN)_6$	KOH wash	None	101
	KOH wash	DBMIB (0.04 μg/ml)	40
	KOH wash	DCMU (3 μg/ml)	0
	KCN wash	None	85
	KCN wash	DBMIB (0.04 μg/ml)	65
	KCN wash	DCMU (3 μg/ml)	0

[a] The reaction mixture for the $H_2O \rightarrow$ferricyanide reaction was as in Table I except no DBMIB was present; the $H_2O \rightarrow$MV reaction was as in Table II, the ascorbate + TMPD→MV reaction contained in 1.5 ml: chloroplasts (50 μg), 37.5 m moles Trizma-Mes, pH 8.0, 50 μmoles sodium ascorbate, 1.2 μmoles DCMU, and 0.1 mg TMPD.

[b] μequiv/mg chlorophyll/h.

[c] PC, plastocyanin.

Cramer [2] have shown that ferricyanide can react with cytochrome *f*. In this study it has also been found that ferricyanide can accept electrons at several sites in PS II Figs 1–4 and Table III).

It has been proposed that dichlorophenyl–dimethylurea and orthophenanthroline inhibit electron flow at the same site [9, 10] in PS II. In this study, however, it has been found that in the presence of OP ferricyanide can accept electrons at two sites in PS II at different pH's (Figs 1–4). In the presence of DBMIB ferricyanide reduction was found to be more insensitive to OP at low pH, whereas at pH 7.5 with DBMIB ferricyanide reduction was found to be completely inhibited by OP. The above data imply that two different sites may be involved in DCMU and OP inhibition in PS II as measured by ferricyanide reduction. Other PS II reactions, such as $H_2O \rightarrow$DMBQ, and $H_2O \rightarrow$MV and the $H_2O \rightarrow$cobaltinitrite reactions, which involve both photosystems showed a different pattern of OP inhibition (Table II). At both pH 5.5 and 8.0 more inhibition of quinone reduction in the presence of OP was observed. The

$H_2O \rightarrow$cobaltinitrite reaction also gave strong OP inhibition at low pH, showing that orthophenanthroline can get to its site of action at low pH.

If the site of ferricyanide reduction in PS I is at P_{700} or beyond, then removal of plastocyanin should inhibit the reduction. Polylysine, which inhibits plastocyanin [5], stimulates ferricyanide reduction in SN chloroplasts. Bathophenanthroline sulfonate, which was found to inhibit PS I reactions beyond the methyl viologen site [11], does not inhibit ferricyanide reduction. Other treatments that inactivate plastocyanin, such as Tween washes or KCN treatment [7], do not affect ferricyanide reduction in SN chloroplasts (Table III). In Tween-washed chloroplasts $H_2O \rightarrow$MV and ascorbate + TMPD$\rightarrow$MV reactions can be restored by exogenous plastocyanin. In contrast, when exogenous plastocyanin was added to the plastocyanin-depleted chloroplasts, the ferricyanide reduction rate was unchanged.

The above data suggest that ferricyanide is reduced in PS II between plastoquinone and plastocyanin. This view agrees with Horton and Cramer [2], who showed that cytochrome *f* is accessible to oxidation by ferricyanide. It also agrees with Avron [12], who showed a ferricyanide reduction site near cytochrome *f*.

Ferricyanide reduction by chloroplasts, but especially chloroplast sensitivity to orthophenanthroline inhibition at low pH, may be a sensitive tool in determining chloroplast sidedness if ferricyanide is a non penetrating acceptor as postulated [8]. In time-course studies of ferricyanide reduction in the presence of DBMIB, two opposing phenomena were noticed, although only one of these is documented here (Fig. 5). Sometimes very high initial rates of ferricyanide reduction by photosystem II were coupled to higher sensitivity to OP inhibition at pH 5.0 than reported in Table II. In such cases chloroplasts became insensitive to OP inhibition within 4–6 h, a phenomenon explainable by thylakoid vesicle inversion or broken vesicles that return to a normal or resealed state on standing.

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