Cytochrome c Reducing Substances in Photosynthetic Electron Transport

Walter Oettmeier and Wolfgang Lockau

Abteilung Biologie der Ruhr-Universität Bochum (W. Germany)

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Photosynthetic cytochrome c reduction by isolated chloroplasts is mediated by substances with characteristics of a "Cytochrome c Reducing Substance" (CRS). A water soluble extract of ether treated, lyophilized chloroplasts (called $S_{L\text{-eth}}$), related to the primary acceptor complex of photosystem I, has CRS-activity. It contains *p*-coumaroyl-*meso*-tartaric acid. By comparison with model substances, the CRS-activity in chloroplasts is attributed to the iron complex of this compound. A number of other iron complexes and numerous quinones with redox potentials from −220 mV to +300 mV are also shown to possess CRS-activity. The CRS preparation from the blue green alga *Anabaena cylindrica*, as first described by Fujita and Myers, contains Fe^{3+}/EDTA, which has all the chemical and functional properties as reported for CRS.

Photosynthetic cytochrome c (from horse heart) reduction by isolated chloroplasts is stimulated by the addition of a soluble mediator, like ferredoxin. Fujita *et al.* [1–5] reported on a specific "Cytochrome c Reducing Substance" (CRS), obtained from several photosynthetic lamellae systems, which could stimulate cytochrome c reduction. During investigations on components involved in photosynthetic ferredoxin reductions by chloroplasts, we noticed that a fraction obtained from chloroplast lamellae (called $S_{L\text{-eth}}$), which would neutralize an antibody inhibition of photosynthetic ferredoxin reduction, also has CRS-activity [6]. The relation of CRS to $S_{L\text{-eth}}$, FRS ("Ferredoxin Reducing Substance") [7, 8] and ORS ("Oxygen Reducing Substance") [9, 10] has been reviewed [6, 11–14]. The purified CRS-active component of heat-treated $S_{L\text{-eth}}$ has absorption at 260 and 308 nm, its chromophoric group has been identified as *p*-coumaroyl-*meso*-tartaric-acid [15]. The prosthetic group of the CRS, as described by Fujita *et al.* [1–5], has not yet been clarified. The authors suggested a quinone [3].

In order to clarify the role of *p*-coumaroyl-*meso*-tartaric acid in CRS, we prepared a number of model compounds and CRS obtained from *Anabaena cylindrica* according to Fujita and Myers [1, 5] for comparison. The results indicate that indeed quinones as well as a number of iron complexes, including its complex with *meso*-tartaric acid and with EDTA, have CRS-activity. Because the procedure of Fujita *et al.* [1–5] for their CRS preparation included EDTA in all purification steps, any possible native CRS in this preparation is masked by the CRS-activity of its content of Fe/EDTA.

Requests for reprints should be sent to Dr. W. Oettmeier, Abteilung Biologie der Ruhr-Universität, *D-4630 Bochum*, Postfach 2148.

Abbreviations: CRS, Cytochrome c reducing substance, $S_{L\text{-eth}}$, water extract of ether treated, lyophilized chloroplasts [6].

Materials and Methods

Spinach chloroplasts were prepared according to Whatley *et al.* [16], washed three times, frozen in liquid nitrogen and stored until use.

For preparation of stock solutions of the iron complexes, the appropriate amount of $FeCl_3 \cdot 6\,H_2O$ was dissolved in water, the appropriate amount of *meso*-tartaric acid, citric acid, $Na_4P_2O_7 \cdot 10\,H_2O$ and EDTA, respectively, was added, the solution adjusted to pH 8.0 and diluted with water to give final concentrations of 0.01 M Fe^{3+}, 0.02 M *meso*-tartaric acid and citric acid, 0.03 M pyrophosphate and 0.01 M EDTA.

Quinones were dissolved in methanol and diluted with water. The concentration of methanol in the final reaction mixture did not exceed 5%.

The reduction of cytochrome c (from horse heart) was followed spectrophotometrically at 546 nm according to Regitz *et al.* [6]. The reaction mixture contained in 1 ml 0.02 M Tris buffer (pH 8.0) 2.5 mM NH_4Cl, 4 mg bovine serum albumin, 0.2 μmoles cytochrome c and chloroplasts with 5 μg/ml chlorophyll. The reaction was run in air for 8 min. The light intensity was $8 \cdot 10^4$ erg sec^{-1} cm^{-2}. In case of

the iron complexes, red light (cut off filter RG 1, Schott & Gen., Mainz, 610 nm), light intensity $6 \cdot 10^4$ erg sec^{-1} cm^{-2}, was used in order to prevent photochemical autoreduction of Fe^{3+}.

If the reduction from Fe^{3+} to Fe^{2+} was measured directly, cytochrome c was substituted by 1 μmole 2,2′-bipyridine and the formation of the red $[Fe(2,2'\text{-bipyridine})_3]^{2+}$ was monitored at 492 nm.

Oxygen consumption in a Mehler-reaction with CRS from *Anabaena cylindrica* was measured polarographically in a Gilson oxygraph. The reaction mixture contained in 1.7 ml 0.02 M Tris buffer, pH 8.0, 1 mM NH_4Cl, 8 mg bovine serum albumin and chloroplasts with 100 μg chlorophyll.

Quantitative estimation of iron content in CRS from *Anabaena cylindrica* was carried out by atomic absorption spectroscopy (Zeiss PMQ II spectrophotometer with atomic absorption attachment, hollow cathode lamp, $\lambda = 248.3$ nm). For calibration solutions with known iron content were used.

Results

With water as the electron donor, chloroplasts in light reduce cytochrome c to a certain extent. Under the experimental conditions applied, this basal rate of reduction varies from 230 – 470 $\Delta E_{546\,nm}/8$ min, due to the quality of chloroplasts. This basal rate can be highly increased by addition of ferredoxin, as demonstrated by Davenport and Hill [18] and Keister and San Pietro [17] or by addition of heated $S_{L\text{-}eth}$, which contains a CRS-active component [6].

I. Stimulation of photosynthetic cytochrome c reduction by quinones and iron complexes

Quinones are widely applied as Hill-reagents in photosynthetic electron transport [19–21]. They also exhibit powerful CRS-activity, as can be seen in Figs 1 and 2 for anthraquinone-2-sulfonic acid, phenanthrenequinone and several substituted *p*-benzoquinones. The redox potentials are in the range from $E_0' = -220$ mV to $E_0' = +300$ mV.

As is demonstrated in Fig. 3, various iron complexes with *meso*-tartaric acid, citric acid and pyrophosphate as complexing agents also markedly stimulate photosynthetic cytochrome c reduction. Stimulation starts at a concentration of about 0.05 mM.

It is assumed that Fe^{3+} in the complexes is reduced to Fe^{2+} by chloroplasts, which subsequently itself reduces cytochrome c. Reduction of Fe^{3+} can

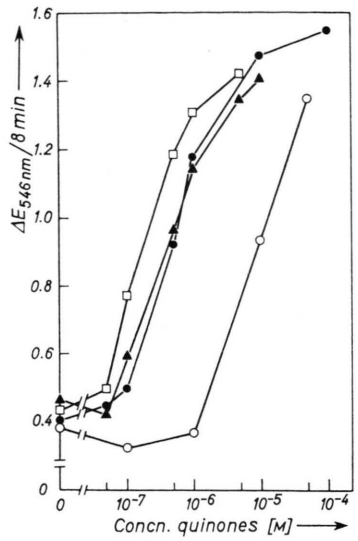

Fig. 1. Stimulation of photosynthetic cytochrome c reduction by quinones. ○—○ Anthraquinone-2-sulfonic acid ($E_0' = -220$ mV), ▲—▲ phenanthrenequinone ($E_0' = -20$ mV), □—□ menadione ($E_0' = -10$ mV), ●—● duroquinone ($E_0' = +70$ mV).

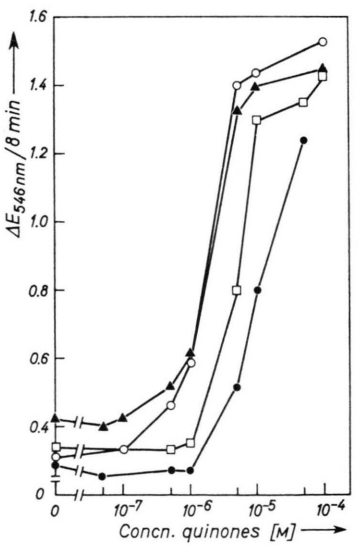

Fig. 2. Stimulation of photosynthetic cytochrome c reduction by quinones. ○—○ 2,3,5-Trimethyl-*p*-benzoquinone ($E_0' = +110$ mV), ▲—▲ 3,6-dimethyl-*p*-benzoquinone ($E_0' = +170$ mV), □—□ toluquinone ($E_0' = +250$ mV), ●—● *p*-benzoquinone ($E_0' = +300$ mV).

be followed directly by substituting cytochrome c by 2,2′-bipyridine, which forms the intense red coloured complex $[Fe(2,2'\text{-bipyridine})_3]^{2+}$. This is shown in Fig. 4 with increasing concentrations of iron *meso*-tartrate.

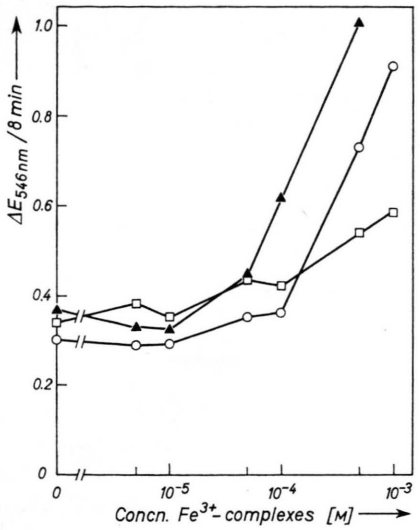

Fig. 3. Stimulation of photosynthetic cytochrome c reduction by iron complexes. ○—○ Fe^{3+}/*meso*-tartaric acid, □—□ Fe^{3+}/citric acid, ▲—▲ Fe^{3+}/pyrophosphate.

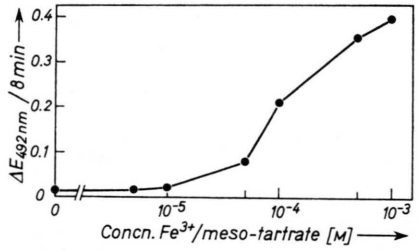

Fig. 4. Formation of $[Fe(2,2'\text{-bipyridine})_3]^{2+}$ upon reduction of Fe^{3+}/*meso*-tartaric acid by illuminated chloroplasts in the presence of 2,2'-bipyridine.

II. On the chemical nature of CRS from Anabaena cylindrica

CRS from *Anabaena cylindrica* was isolated exactly in the same way, as described by Fujita and Myers [1, 5]. The procedure started with sonification of *Anabaena* lamellae systems, acetone treatment, followed by extraction with 0.1 M Tris buffer, pH 7.6, containing 10^{-3} M EDTA. The extract was heated to 100 °C, dialysed and finally chromatographed on a Sephadex G-25 column. The CRS preparation, thus obtained, was identical to that of Fujita and Myers [1, 5], as estimated from the absorption spectrum and stimulation of photosynthetic cytochrome c reduction.

Fujita and Myers [3, 5] have reported also on the autooxidation of CRS, accompanied by photophosphorylation. In Table I, oxygen consumption with

CRS [ml]	O_2 μmoles/h/mg chlorophyll
0	<0.1
0.1	3.8
0.2	9.2
0.4	13.8
1.0	25.8

Table I. Stimulation of oxygen consumption of spinach chloroplasts by CRS from *Anabaena cylindrica*.

CRS in a Mehler-reaction is demonstrated. As can be seen, the stimulation of oxygen consumption is about proportional to the amount of CRS added.

We have, however, observed that a large amount of CRS is lost upon dialysis. This indicated that the molecular weight of 3000 – 5000, as assumed by Fujita and Myers [3], seems to be much smaller.

The UV-spectrum of our preparation of CRS from *Anabaena* (Fig. 5) is identical to that published by Fujita and Myers [1, 5]. We also could ob-

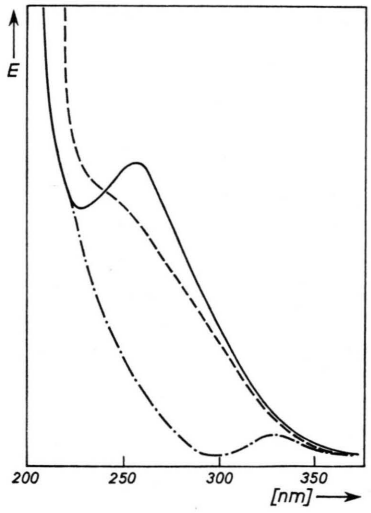

Fig. 5. Absorption spectrum of CRS from *Anabaena cylindrica* ——— in H_2O, — — — after addition of KBH_4, —·—·— in 1 N HCl.

serve the change in absorption upon addition of borohydride, as reported [1, 5]. However, a drastic change of the absorption spectrum of CRS from *Anabaena* is caused by adding hydrochloric acid (Fig. 5). The absorption maximum at 260 nm completely vanishes and a new absorption maximum at 335 nm with low intensity appears. This change can only be observed with hydrochloric acid or other strong acids in the presence of chloride ions. The bathochromic shift from 260 nm to 335 nm cannot be interpreted considering CRS as a quinone, whose absorption spectrum would remain unchanged under

these conditions. It seems more obvious that the new absorption maximum at 335 nm is due to the formation of an iron chloro-complex. In fact, the presence of Fe^{3+} in CRS from *Anabaena* was proved by formation of red $Fe(SCN)_3$ upon addition of SCN^- in acidic medium as well as by atomic absorption spectroscopy.

Furthermore, there is a linear correlation between optical density at 260 nm and iron content. Different preparations of CRS always exhibit a constant ratio $E_{260\,nm}$ versus μg Fe/ml of 0.106 ± 0.009. These results strongly indicate that CRS from *Anabaena* has to be considered as an iron complex. As to the ligand, we realized that *Anabaena* lamellae systems have been extracted by 0.1 M Tris buffer containing 10^{-3} M EDTA. The ligand therefore could be EDTA.

Comparison of Fe^{3+}/EDTA and CRS in thin layer chromatography on different layers and in different solvent systems (Table II) revealed the identity of both. In addition, the absorption spectra of Fe^{3+}/EDTA and CRS from *Anabaena* (provided equal Fe^{3+} concentrations) are identical in shape and optical density at 260 nm as in Fig. 5 and also in flattening of the maximum at 260 nm upon addition of borohydride and the bathochromic shift of the maximum to 335 nm in 1 N HCl.

Table II. Comparison of Fe^{3+}/EDTA and CRS from *Anabaena cylindrica* in thinlayer chromatography.

Solvent system		R_F Fe^{3+}/EDTA	R_F CRS
CH_3OH/H_2O 1:1	(a)	0.42	0.42
CH_3OH	(b)	0.61	0.57
CH_3OH/H_2O 4:1	(b)	0.67	0.66
15% CH_3COOH	(b)	0.87	0.86

(a) Silica gel, (b) cellulose. Spots were detected by spraying with Na-ascorbate/2,2′-bipyridine.

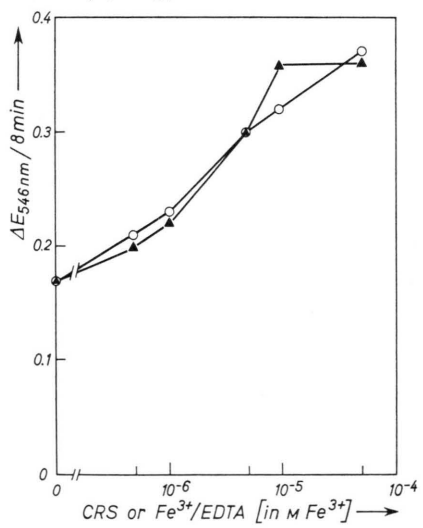

Fig. 6. Comparison of Fe^{3+}/EDTA and CRS from *Anabaena cylindrica* in photosynthetic cytochrome c reduction. ○—○ Fe^{3+}/EDTA, ▲—▲ CRS from *Anabaena*.

Fe^{3+}/EDTA and CRS from *Anabaena* were compared in their ability of stimulating photosynthetic cytochrome c reduction. As is shown in Fig. 6, both CRS and Fe^{3+}/EDTA (at the same Fe-titer) are equally stimulating cytochrome c reduction.

Discussion

The redox potential of cytochrome c is fairly electropositive ($E_0' = +260$ mV). As known, numerous compounds with a more electronegative potential reduce cytochrome c chemically. If such compounds are themselves reduced by chloroplasts in the light, it is to be expected that these compounds mediate cytochrome c reduction by chloroplasts in the light, *i. e.* they exhibit CRS-activity. Indeed, a variety of substances, commonly used as Hill-reagents, were found to stimulate photosynthetic cytochrome c reduction, like pyocyanin, phenazine methosulphate, ferricyanide, benzyl- and methylviologen, FMN and 2,3′,6-trichlorophenol-indophenol [17].

Ferredoxin, however, was the first natural constituent of the photosynthetic electron transport chain, to be shown to catalyse photosynthetic cytochrome c reduction, as demonstrated by Davenport and Hill [18] and Keister and San Pietro [17]. A second natural cytochrome c reducing substance, quite distinct from ferredoxin, seemed to have been discovered by Fujita and Myers [1] in the blue green alga *Anabaena cylindrica* and later in a great variety of algae and green plants [2] and is commonly referred to as CRS. As reported by Fujita and Myers [1], CRS is heat stable, has a molecular weight of about 3000 – 5000 and a broad absorption maximum at 260 nm. Fujita and Myers [3] suggested that CRS might be a quinone.

As the results presented indicate, indeed a great variety of quinones exhibit CRS-activity, as has to be expected, because quinones as Hill-reagents in photosynthetic electron transport have been extensively used [19–21] and because certain hydroquinones are routinely used as electron donors at the cytochrome c level in mitochondria as well as in chloroplasts. Vernon and Shaw [22] have already found that trimethylbenzoquinone and ubiquinone have CRS-

activity. In addition, quinones, covering the redox potentials from -220 mV to $+300$ mV are shown to have CRS-activity; surprisingly also benzoquinone ($E_0' = +300$ mV). However, calculation by the Nernst-equation reveals that the redox potential of the system benzoquinone/benzohydroquinone, with an excess of benzohydroquinone present, is lowered so much that reduction of cytochrome c can occur. Besides, benzohydroquinone reduces cytochrome c chemically.

As reported by Regitz *et al.* [6], a heated water extract from ether treated lyophilized chloroplasts – called $S_{L\text{-eth}}$ – carrying antigen activity against an antibody against the primary acceptor complex of photosystem I, also is active in stimulating cytochrome c reduction. Our results indicate that CRS from spinach is related to a compound containing *p*-coumaroyl-*meso*-tartaric acid [11, 12, 15]. As the results, presented in this paper, demonstrate, several iron complexes of *meso*-tartaric acid, citric acid and pyrophosphate are capable of catalysing cytochrome c reduction. Therefore, we assume that a metal complex, possibly iron, with *p*-coumaroyl-*meso*-tartaric acid, is responsible for the CRS-activity of $S_{L\text{-eth}}$.

As to the mechanism of reduction, Fe^{3+} is reduced to Fe^{2+}, which subsequently reduces cytochrome c. The direct reduction of Fe^{3+} to Fe^{2+} has been proved by the formation of the Fe^{2+}/2,2′-bipyridine complex (Fig. 4).

In addition to these iron complexes, also Fe/EDTA exhibits excellent CRS-activity. By following the procedure of Fujita and Myers [1, 5], one obtains a compound, which is identical with Fe/EDTA, as jugded by spectroscopic, chromatographic and functional properties. Fe^{2+}/EDTA is also autooxidizable. This autooxidation accounts for oxygen consumption in a Mehler-reaction with Fe^{3+}/EDTA as the acceptor.

Fujita *et al.* [1–5], in describing the properties of CRS from various sources always included EDTA in the culture medium as well as in the purification steps, possibly for safety reasons. It is not surprising, therefore, that one cannot avoid isolating Fe/EDTA. Therefore, CRS from *Anabaena cylindrica* has to be considered as an artefact. It is quite likely that CRS preparations from other origins by Fujita *et al.* again are identical with Fe/EDTA, since the applied extraction procedures are the same as in the case of *Anabaena*. This does not exclude though, that in the preparations by Fujita *et al.* Fe/EDTA is masking a native CRS.

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