Fructose-Stimulated Ferricyanide Reduction by Intact Blue-Green Algae

Siegfried Scherer and Peter Böger

Lehrstuhl für Physiologie und Biochemie der Pflanzen, Universität Konstanz, D-7750 Konstanz, Bundesrepublik Deutschland

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As reported in the literature, ferricyanide reduction of blue-green algal cells in the dark is linked to respiratory electron transport. This study has not verified this hypothesis. There was no concurrent activity of ferricyanide reduction by intact cells with respiratory oxygen uptake. It was found light-independent, showing little KCN-inhibition, but was strongly CCCP-sensitive. CCCP inhibition was reversed by KCN. – Both, respiration and ferricyanide reduction were stimulated by fructose which was actively accumulated by the cells (the apparent K_m was 20 μM). Ferricyanide reduction was more sensitive towards dark starvation than respiration. Based on these data it is suggested that ferricyanide reduction and cyanide-sensitive respiration are parts of different electron-transport processes, competing for reduction equivalents originating from the same (endogenous) carbon pool.

Introduction

Literature is controversial as to whether cyanobacterial respiratory electron transport is located on the cytoplasmic membrane [1]. Murata [2] and Lockau [3], having isolated a cytoplasmic membrane fraction from *Anacystis* and *Anabaena*, respectively, failed to demonstrate cytochrome aa_3 or cytochrome-oxidase activity present in these membranes. In contrast, Peschek and coworkers reported evidence for a cytochrome-$a\,a_3$ oxidase located on the plasma membrane of *Anacystis* (for review see [4]).

Recently, Craig and coworkers [5] showed that intact *Anacystis* cells were able to reduce ferricyanide, a reaction common to many plasma membranes [6]. They concluded exogenous ferricyanide reduction as being indicative of a respiratory chain located on the cytoplasmic membrane of *Anacystis*. In the study presented here, we report on ferricyanide reduction of intact cells of *Anabaena variabilis* concluding that this reaction apparently is not necessarily linked to respiratory electron transport.

Abbreviations: ATCC, American Type Culture Collection, Rockville, Md, USA; BPDS, bathophenanthroline disulphonate; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; Chl, chlorophyll *a*; EDTA, ethylenediamine tetraacetic acid; HOQNO, 2-*n*-heptyl-4-hydroxyquinoline-N-oxide; SAUG, Algae Culture Collection, University of Göttingen, Germany; TTFA, 4,4,4-trifluoro-1-(2-thienyl)-1,3-butanedione.

Reprint requests to Prof. Dr. P. Böger.

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Materials and Methods

Anabaena variabilis Kütz. (ATCC 29413), *Aphanocapsa* (ATCC 27178), *Phormidium foveolarum* (SAUG B-1462-1) and *Mastigocladus laminosus* (SAUG B-4.84) were grown as described [7]. Cells were harvested at various stages of growth and resuspended in 50 mM sodium-phosphate buffer, pH 7.0. Chlorophyll was determined according to [8], oxygen uptake was measured by a Clark-type electrode at 24 °C.

The assay for potassium ferricyanide reduction contained 0.4 mM ferricyanide and was performed at 24 °C in 38-ml vials in the dark or in the light, respectively, as indicated (450 $\mu E/m^2 \times s$ at the bottom of the vials, using simple tungsten bulbs). Within a 100 min reaction time, several aliquots were withdrawn to establish a kinetics of ferricyanide reduction. After centrifugation (Eppendorf, model 5412), the absorbance of the supernatant was measured at 420 nm before and after the addition of a few grains of sodium dithionite. The remaining ferricyanide content after biological reduction was calculated from the absorbance difference using an extinction coefficient of 1 $mM^{-1} \times cm^{-1}$. Ferricyanide reduction caused by a supernatant of *Anabaena* cells alone, preincubated without ferricyanide for 60 min, was negligible.

Determination of Fe^{3+}-reduction was performed at 24 °C according to Chaney *et al.* [9], using 0.15 mM BPDS and 0.05 mM $FeCl_3$-EDTA. Aliquots were withdrawn and centrifuged at the time intervals indicated. Formation of the ferrous BPDS complex was measured in the supernatant at 535 nm using an extinction coefficient of 22 $mM^{-1} \times cm^{-1}$ [10].

All rates of ferricyanide and Fe^{3+}-reduction were calculated from the slope of a kinetics curve, each comprising 5 to 7 measurements.

Chemicals, analytical grade, were purchased from Merck, Darmstadt, Germany. BPDS, CCCP, HOQNO, and TTFA were from Sigma, Taufkirchen, Germany.

Results and Discussion

In our hands, *Aphanocapsa*, *Phormidium* as well as *Anabaena* cells reduced externally added ferricyanide (data not shown), thus confirming that apparently an appropriate dehydrogenase is located on the cytoplasmic membrane, as reported by Craig *et al.* [5] using *Anacystis*. We checked for a possible correlation between respiratory electron transport and ferricyanide reduction in *Anabaena variabilis*. During batch cultivation, respiratory activity changed markedly (Fig. 1, B), furthermore we found that old *Anabaena* cultures exhibited a higher ferricyanide reduction as compared to young cultures (part A). Our data clearly show that there is no correlation of ferricyanide reduction with respiration (the same result was obtained with *Mastigocladus*, data not shown). In Fig. 1 A, ferricyanide reduction is also expressed as percent of respiration (■—■), demonstrating more clearly different activity of the two processes during cultivation time, since in this case no reference is made to the chlorophyll content varying with the age of the culture. If ferricyanide reduction were due to a *respiratory* dehydrogenase and/or redox-active quinone, one would expect that the time course of ferricyanide reduction correlates with cytochrome-oxidase activity, *i.e.* oxygen uptake. Evidently, this is not the case.

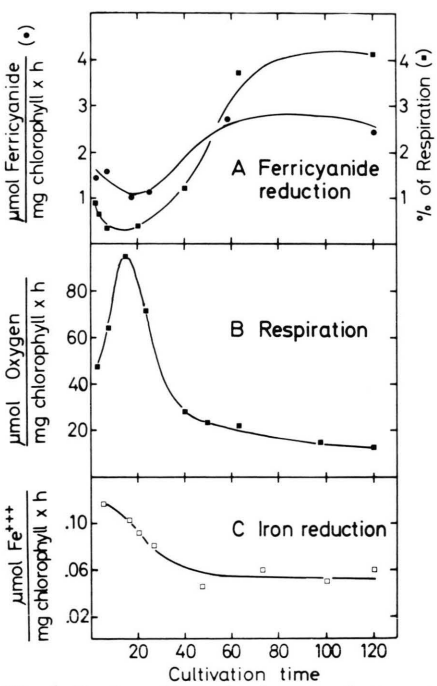

Fig. 1. Ferricyanide reduction, respiratory oxygen uptake and iron reduction as a function of the age of an *Anabaena* batch culture. The data represent average values calculated from 5 to 10 experiments. To estimate the ferricyanide reduction in percent of respiration it was assumed that 4 µmol potassium ferricyanide reduced corresponds to 1 µmol oxygen taken up.

It has been claimed that oxygen-dependent proton efflux of *Anacystis* is due to a cytochrome oxidase and a respiratory electron transport located on the cytoplasmic membrane [13]. If ferricyanide reduction received electrons from such an electron-transport chain, rates should be in about the same order of magnitude for both proton efflux and ferricyanide reduction. As demonstrated in Fig. 1 A, the ferricyanide reduction of a two-day batch culture is about 1 to 2 µmol/mg Chl × h. A H^+ to e ratio of 2 to 4 is commonly found in bacteria (for reviews see [1]). From both ferricyanide reduction and the H^+/e-values we calculate a proton efflux of 2 to 8 µmol H^+/mg Chl × h. The observed proton efflux of *Anabaena* under these conditions, however, is 30 to 60 µmol/mg Chl × h [1], which is one order of magnitude higher.

There is evidence for higher plants that ferricyanide reduction of cytoplasmic membranes is due to an electron-transport system [11], possibly connected with iron reduction [12], which seems to be a prerequisite of iron uptake [9]. Therefore, we tried to relate ferricyanide reduction with iron reduction (Fig. 1 C). No correlation was found either. Iron-starved cultures neither exhibited stimulated ferricyanide or stimulated iron reduction (data not shown). So, at the moment, no conclusive data are available as to whether the redox process(es) measured by ferricyanide reduction is (are) connected to iron uptake mechanisms.

Ferricyanide reduction as well as respiratory oxygen uptake can be stimulated markedly by fructose, using starved cells (Fig. 2). It has been shown previously that the particular *Anabaena* strain used in this study can grow with fructose in the dark [14], and that nitrogenase activity is stimulated by fructose [15]. A K_m of 20 µM for fructose is apparent (Fig. 3) indicative of an active transport mechanism (comp. [16]). The latter evidence can be further supported by applying an uncoupler (Fig. 4), since CCCP prevents fructose-stimulated oxygen uptake. From the stimulation of both oxygen and ferricyanide reduction we conclude that both processes are connected to the same fructose-uptake

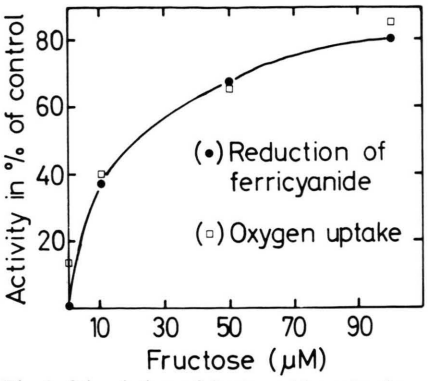

Fig. 2. Stimulation of ferricyanide reduction and respiratory oxygen uptake by added fructose. 100% of control are equivalent to the reaction under fructose-saturated conditions, corresponding to 4.7 μmol ferricyanide/mg Chl × h and 70 μmol O_2/mg Chl × h. The cells used in this experiment were incubated under shaking for 18 h in the dark, without fructose, at 24 °C.

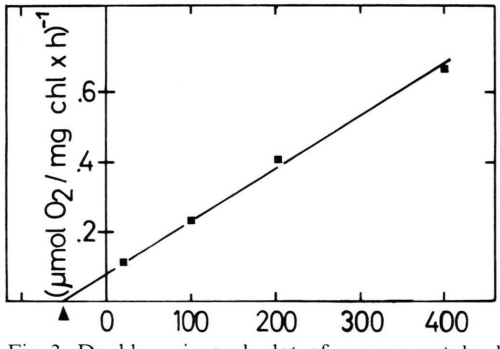

Fig. 3. Double-reciprocal plot of oxygen uptake by *Anabaena* versus fructose concentration using the data given in Fig. 2.

Table I. Effect of several inhibitors on reduction of ferricyanide by *Anabaena*.

Additions	Activity in % of dark control, ± σ	Number of experiments
Control	100	
In the light, no additions	112 ± 24	13
(+) TTFA, 10^{-4} M	89 ± 10	5
(+) HOQNO, 10^{-5} M	97 ± 7	4
(+) KCN, 10^{-3} M	80 ± 8	7
(+) CCCP, 10^{-5} M	48 ± 13	7
(+) CCCP, 10^{-5} M, plus KCN, 10^{-3} M	83 ± 9	4

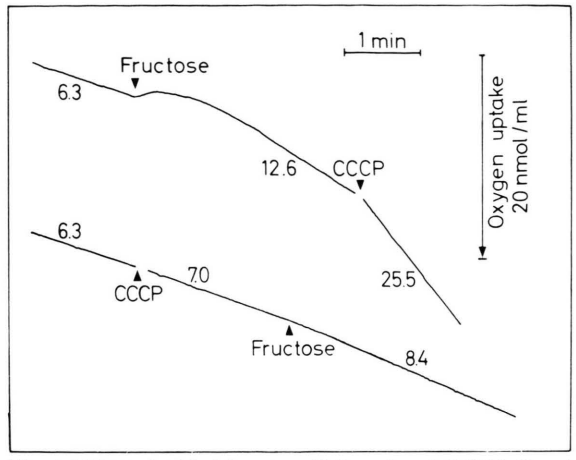

Fig. 4. Effect of added fructose (0.5 mM) and CCCP (6 μM) on respiratory oxygen uptake by *Anabaena* cells, incubated for 6 h in the dark prior to the experiment. The figures represent oxygen-uptake rates in μmol O_2/mg Chl × h, the reaction chamber contained 22 μg Chl/ml.

and -dissimilation pathway, most possibly yielding reduced pyridine nucleotides as reducing substrates. Noteworthy, under starving conditions, ferricyanide reduction was not detectable long before oxygen uptake decreased to zero (Fig. 2; see zero fructose: (○) and (□) symbols). This again points to different regulatory features of the two processes, at least under carbon-limited conditions.

The effect of several inhibitors on ferricyanide reduction is shown in Table I. Light did not substantially influence ferricyanide reduction, in accordance with the data of Craig *et al.* [5]. TTFA, known to affect mitochondrial dehydrogenases, only slightly inhibited ferricyanide reduction. Stimulation of the latter by HOQNO was reported [5], which inhibits electron flow at the cytochrome-*b*/*c* complex. This finding was interpreted as being indicative of ferricyanide competing with the cytochrome-*b*/*c* complex for electrons donated by the same dehydrogenase. We have not been able to reproduce this effect using up to 10 or 80 μM HQNOQ. If such a competition were operative, KCN should stimulate ferricyanide reduction as well, but – in disagreement with [5] – we observed a significant inhibition. The uncoupler CCCP on the one hand inhibited ferricyanide reduction markedly and, on the other hand, stimulated respiratory oxygen uptake (comp. Fig. 4). This uncoupler inhibition was reversible by adding KCN to the CCCP assay.

Summarizing then, our results are as follows: (a) There is a clear negative correlation between respiration and ferricyanide reduction; (b) respiratory oxygen uptake is definitely more resistant towards starvation than ferricyanide reduction; (c) when stimulating respiratory electron transport with CCCP, ferricyanide reduction is decreased, and (d) both ferricyanide reduction and respiration are stimulated by added fructose, *i.e.* both are coupled to the same endogenous carbon pool. Craig *et al.* [5] interpreted the ferricyanide reduction of intact *Anacystis* cells as being due to a respiratory electron-transport chain located on the cytoplasmic membrane. In contrast, we suggest that respiratory electron transport and ferricyanide belong to different redox processes competing for the same pool of reduction equivalents. Respiration, however, apparently being more important for the cell, is preferably provided with reduction equivalents.

The physiological role and nature of the ferricyanide-reducing redox system apparently located on the cytoplasmic membrane are not yet clear. Most interestingly in this respect, Murata and coworkers isolated cytoplasmic membranes from *Anacystis* (and *Anabaena*) carrying NAD(P)H dehydrogenases not associated with a cytochrome oxidase (N. Murata, 1984, personal communication), but with one or two low mid-point potential cytochromes different from the thylakoidal cytochromes [2].

Acknowledgements

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