Extracellular ferricyanide reduction and nitrate reductase activity in the green alga *Monoraphidium braunii*

Alfonso Corzo\textsuperscript{a}, Reiner Plasa\textsuperscript{b} and Wolfram R. Ullrich\textsuperscript{b}

\textsuperscript{a}Departamento de Ecología, Universidad de Málaga, Campus Universitario de Teatinos, 29071 Málaga (Spain) and \textsuperscript{b}Institut für Botanik, Technische Hochschule, Schnittpahnstrasse 3, D-6100 Darmstadt (Germany)

(Received November 15th, 1990; revision received January 9th, 1991; accepted January 21st, 1991)

The unicellular green alga, *Monoraphidium braunii*, reduced extracellular ferricyanide at high rates. Impermeability of the membranes to ferri- and ferrocyanide was shown for untreated cells as for those permeabilized with 5% 1-propanol. Fe starvation stimulated reduction rates by 50%, light or glucose were ineffective. Extracellular acidification largely corresponded to formation of the new anionic valence of ferrocyanide. 1-Propanol used as permeabilizer for NR assays in the same samples apparently inhibited ferricyanide reduction, however addition of catalase showed that this was not due to interference with ferricyanide reduction but to partial reoxidation by \(H_2O_2\). Between ferricyanide reduction and nitrate reduction no competition was found in an ‘in situ’ system with endogenous reductant in the presence of 1-propanol, neither with ‘induced’ algae showing high NR activity nor with ‘repressed’ algae grown in ammonium medium and showing only constitutive NR activity. According to this data the inducible high-activity NR and the inducible nitrate uptake system are not responsible for reduction of ferricyanide. The plasmalemma-bound, low activity, NR might have extracellular reducing activity, but also for this NR lack of competition in reduction of the two substrates indicated independent reactions in *Monoraphidium*.

Key words: ferricyanide; extracellular reduction; nitrate reductase; *Monoraphidium braunii*

Introduction

Reduction of ferricyanide by intact unicellular algae has been reported by Good [1] and later by Novak and Miklashevich [2] and Nešpůrková et al. [3], reduction of ferrooxamine B and ferric citrate more recently by Allnutt and Bonner [4]. Reducing power for this rapid iron reduction has been found to be derived from photosynthesis, tricarboxylic or oxidative pentose phosphate cycles. This extracellular reduction has been studied with various electron acceptors in higher plants or algae, often with ferricyanide (hexacyanoferrate III) as the electron acceptor [5—9].

An essential question of this process has been so far, and still is, its physiological and ecological role. Jones et al. [10] for phytoplankton, as others for roots, assume that it is mainly used for solubilizing and absorbing trace elements, such as iron and manganese, but this function would not require the high rates measured in some plant or algal materials.

Since Butz and Jackson [11] proposed a plasmalemma-bound nitrate reductase being also responsible for nitrate uptake, the functioning of such an enzyme has been supported and doubted by various experimental data [8,12—14]. Based on immunological techniques, the idea has been experimentally supported. A constitutive part of the nitrate reductase found in cells of *Chlorella* and in roots has been shown to be attached to the plasmalemma, and addition of anti-nitrate-reductase antibodies could also inhibit nitrate transport, in isolated protoplasts as well as in...
whole cells [15–17]. Reduction of siderophores by nitrate reductase, but at much lower rates than nitrate reduction, was demonstrated by other groups [18]. With the same techniques but in the diatom *Thalassiosira*, Jones and Morel [19] showed a triangular immunological correlation between NR activity, nitrate uptake and extracellular ferricyanide reduction. NR antibody was detected to (1) bind to the cell surface by using immunofluorescent labelling, (2) to inhibit the plasmalemma redox activity in this organism and (3) to inhibit primary amine production in the presence of the impermeant electron acceptor Cu<sup>2+</sup>-bathophenanthroline disulfonate. Jones and Morel’s hypothesis proposed according to these data implies a direct competition for electrons originating from NAD(P)H between nitrate reduction and extracellular ferricyanide reduction, both being assumed to be activities of the same plasmalemma redox chain.

The present paper reports on extracellular ferricyanide reduction activities of *Monoraphidium* focusing mainly on the relationship between the activities of nitrate reductase and the plasmalemma redox system. To enable measurements of ferricyanide reduction and NR activity in situ, a method was developed to eliminate the interference of ferri- and ferrocyanide with nitrite determination by reducing ferricyanide with ascorbate and precipitating the formed ferrocyanide with Zn<sup>2+</sup>.

**Material and Methods**

**Material**

*Monoraphidium braunii*, strain 202-7d, from the algal collection in Göttingen was cultured synchronously with a 14/10-h light/dark rhythm at 29°C in a medium with the main nutrients: 8 mM KNO<sub>3</sub>, 8 mM NaNO<sub>3</sub>, 0.1 mM Ca(NO<sub>3</sub>)<sub>2</sub>, 1 mM MgSO<sub>4</sub>, 3.4 mM Na<sub>2</sub>HPO<sub>4</sub>, 0.8 mM Na<sub>2</sub>HPO<sub>4</sub>, zinc, iron, and trace elements, pH 6.2. For the experiments, cultures were taken from the exponential growth phase. Where indicated, the cells had been cultured without iron and trace elements for 3 days.

**NR assay in situ**

A 1-ml sample of the algal suspension (cell densities 20–40 μg chlorophyll ml<sup>−1</sup> for NO<sub>3</sub>−-grown, 60–90 μg chl ml<sup>−1</sup> for NH<sub>4</sub>+-grown cells) was added to 1 ml of an assay mixture with 200 mM KNO<sub>3</sub>, 100 mM K-phosphate buffer (pH 7.5) and 10% 1-propanol (i.e. double final concentrations). Five percent of 1-propanol were found to be necessary for *Monoraphidium*. The samples were incubated in the dark at 29°C. The reaction was stopped at several points during the linear reaction phase (0–20 min) by filtration through membrane filters (cellulose acetate, 0.8 μm pore size, Sartorius, Göttingen, F.R.G.). The generated nitrite was determined immediately by photometry of an azo dye [20].

This assay is called ‘in situ’ (not ‘in vivo’) in this article in spite of some different use of these terms [21], because the algae are used in a ‘premortal’ rather than in a living state, while the enzyme is assayed at its natural place within the cells (in situ).

**Ferricyanide reduction assay**

A 1-ml sample of algal suspension in the culture solution was added to 1 ml of a mixture containing 10 mM CaSO<sub>4</sub>, 30 mM Mes-Tris buffer (pH 7.5) and 2 mM ferricyanide as K<sub>3</sub>Fe(CN)<sub>6</sub>. The samples were incubated in the dark at 29°C for 15 min, and the reaction was stopped by filtration. Disappearance of ferricyanide in the filtrate was measured at 420 nm.

**Ferricyanide and nitrate reduction in the same samples**

Prior to the experiment (30–60 min) the algae were filtered, resuspended in 5 mM CaSO<sub>4</sub> and kept in the standard culture conditions. The experiments were carried out with an algal cell density of 20–40 mg chl l<sup>−1</sup> (algae grown in nitrate) and 60–90 mg chl l<sup>−1</sup> (algae grown in ammonium) in a medium containing 100 mM KNO<sub>3</sub>, 50 mM phosphate buffer (pH 7.5), 2.5 mM CaSO<sub>4</sub> and 5% (v/v) 1-propanol. At the start of the experiments or after 10 min, ferricyanide was added to reach a final concentration of 1 mM. Control samples were run without ferricyanide. All experiments were performed in the dark at 29°C. Samples were removed at intervals, filtered through membrane filters, and the remaining ferricyanide was measured at 420 nm. Both ferri-
cyanide and ferrocyanide interfere with the formation of the azo dye required for the determination of nitrite and, hence, had to be removed prior to addition of the respective reagents. This was achieved by adding 10 µl of 0.1 M fresh ascorbic acid (to reduce ferricyanide to ferrocyanide) and 50 µl of 0.1 M ZnSO₄ (to precipitate the insoluble Zn₂Fe(CN)₆) to 1 ml of the sample. After centrifugation at 4000 rev./min for 15 min nitrite was measured in the supernatant. A comparison between the efficiency of the original method of nitrite determination and after addition and precipitation of ferrocyanide is shown in Fig. 1.

**Extracellular acidification**

It was determined by titration of the samples with 25 mM KOH at the end of the experiments. The original pH was 5.0 in these cases to avoid interference by HCO₃⁻.

**Chlorophyll**

Total chlorophyll was determined with 1-ml samples according to Senger [22] with hot methanol or, after extraction of the boiled algae, with 80% acetone.

**Statistics**

Figures and tables show either means of n experiments or single representative experiments out of a group of replicates (n) as indicated.

**Results**

*Monoraphidium braunii*, similar to other algal and higher plant cells, reduced ferricyanide at considerable rates (Table I). Starvation of iron for 3 days caused an increase in ferricyanide reduction rates of more than 50%. Reduction was accompanied by an extracellular acidification that was a little stronger than accounted for by the generation of the new anionic valence of ferrocyanide (H⁺/e⁻ = 1.16—1.17; Table I). Addition of glucose, which was easily taken up by *Monoraphidium* and could provide more reducing power [23], did not stimulate ferricyanide reduction in the dark. In the light, ferricyanide reduction was scarcely

**Table 1.** Rates of ferricyanide reduction by *Monoraphidium braunii* in the dark under different conditions, pH 6.2. Rates in µmol mg⁻¹chl h⁻¹. Average rate (Fe-starved) 28.1 (n = 16), maximum 56.0, minimum 9.3. n, number of experiments. Values for comparison only from same experiments.

<table>
<thead>
<tr>
<th>Additions</th>
<th>[Fe(CN)₆]³⁻ reduced</th>
<th>Acidification</th>
<th>Ratios</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fe-unstarved</td>
<td>8.8 (2)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fe-starved</td>
<td>13.8 (2)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fe-starved + glucose</td>
<td>44.7 (4)</td>
<td>52.3 (7)</td>
<td>H⁺/e⁻ 1.17</td>
</tr>
<tr>
<td></td>
<td>−glucose</td>
<td>46.5 (4)</td>
<td>H⁺/e⁻ 1.16</td>
</tr>
<tr>
<td></td>
<td>K₃Fe(CN)₆</td>
<td>22.1 (4)</td>
<td>K⁺/Na⁺ 1.10</td>
</tr>
<tr>
<td></td>
<td>Na₃Fe(CN)₆</td>
<td>20.2 (2)</td>
<td></td>
</tr>
</tbody>
</table>

*Direct comparison only; with indirect comparison, 25 experiments with unstarved, 16 with Fe-starved cells gave the same results.*
stimulated (data not shown) but a rapid decomposition of ferrocyanide to free cyanide performed by the algae (not by a cell-free system) was observed and led to the formation of blue Fe-complex with the remaining ferricyanide.

As known from other plant material, the plasmalemma is usually impermeable to ferricyanide so that its reduction must occur extracellularly. Since the in situ NR assay requires the use of a membrane permeabilizer, it became necessary to show that ferricyanide reduction was also extracellular under these conditions, i.e. in the presence of 5% 1-propanol, the most effective permeabilizer in *Monoraphidium* (Plasa, unpublished results). Whole cells of *Monoraphidium* were incubated in a solution containing 5 mM CaSO₄, 50 mM phosphate buffer (pH 7.5), 5% 1-propanol and 1 mM ferricyanide for 15, 30, 45 and 60 min. The samples were then filtrated and washed with the same, but ferricyanide-free, medium. The cells were deep-frozen with liquid N₂, disintegrated with a Microdismembrator (Braun, Melsungen, F.R.G.), resuspended with distilled water in an Ultra-Turrax and centrifuged at 4000 rev./min. The supernatant was used for assays of both ferricyanide with Fe²⁺, and ferrocyanide with Fe³⁺. Figure 2 shows that the total amount of ferricyanide used was recovered in the cell-free medium in the presence, in this case after reoxidation with H₂O₂, as well as in the absence of 1-propanol. This was confirmed with algal cells. After their disruption, no formation of Fe-ferricyanide complexes could be found with either Fe²⁺ or Fe³⁺, even after several hours of in vivo exposure to ferricyanide (n = 8).

In the presence of 1-propanol the rate of ferricyanide reduction appeared to be even to 40% lower than in its absence. Addition of catalase to the medium completely abolished this ‘inhibition’ (Fig. 3). Hence, 1-propanol apparently caused an increase in formation of H₂O₂, which partly reoxidized the newly formed ferrocyanide and thus led to an underestimation of the ferricyanide reduction rate in its presence. This result was confirmed by kinetic analysis. In the absence of 1-propanol the approximate kinetic parameters of external ferricyanide reduction in the dark were: $K_m = 0.12$ mM and $V_{max} = 26$ µmol Fecy mg⁻¹ chl h⁻¹. The effect of 1-propanol simulated non-competitive inhibition: the apparent $V_{max}$ was much lower by reoxidation of ferrocyanide with H₂O₂, but $K_m$ remained almost unchanged.

According to this evidence, ferricyanide reduction in the presence of 5% 1-propanol (the optimum concentration for NR assays in *Monoraphidium*) proceeded at full rates.
Therefore, the experimental system used here was adequate to reveal a possible competition between extracellular ferricyanide reduction and internal or membrane-bound nitrate reduction. However, the addition of ferricyanide at three different concentrations to nitrate reducing algae (grown on NO₃⁻) did not change the rate of NO₂⁻ production either in a shorter or longer period of time (Figs. 4A and 4B). Both substrates were reduced simultaneously without apparent interference, in spite of the high rates (Fig. 4B).

According to the data from Chlorella and barley roots, two different isoenzymes of NR have been proposed, one cytoplasmic and inducible, the other, with much lower activity, located in the plasmalemma and constitutive, because it also functions in Chlorella cells grown in NH₄⁺ media [15,16]. In Monoraphidium this constitutive NR remained active when the cells had been grown with 8 mM NH₄Cl as the nitrogen source and represented 5—8% of the total activity of NO₃⁻-grown cells. Therefore, the true competition between ferricyanide and nitrate reduction at the plasmalemma enzyme could have been concealed in the presence of cytoplasmic NR activity. However, as in nitrate-grown cells, the rate of NO₂⁻ formation in NH₄⁺-grown cells did not show any inhibition by ferricyanide, when added either at the onset of the experiment or 10 min later (Figs. 5A and 5B). In both cases, ferricyanide even caused an increase in the NO₂⁻ formation rate, in contrast to the data obtained with Thalassiosira [19].

To exclude all kinds of competition also the inverse experiments were carried out. The rate of ferricyanide reduction in Monoraphidium braunii was not altered, even slightly stimulated, by KNO₃ up to 5 mM (Table II). A weak inhibition

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**Fig. 4.** Time course of NO₂⁻ formation in NO₃⁻-grown cells with and without ferricyanide (added after 10 min, see arrows), in the presence of 5% 1-propanol (representative experiments, n = 4). (A) At different ferricyanide concentrations: no [Fe(CN)₆]³⁻ (○), 0.1 mM (●), 0.5 mM (■), 1.0 mM (♦). (B) In comparison with the time course of [Fe(CN)₆]³⁻ reduction: NO₂⁻ no [Fe(CN)₆]³⁻ (○); NO₂⁻, 1 mM [Fe(CN)₆]³⁻ (●), unreduced ferricyanide (○).

**Fig. 5.** Time course of NO₂⁻ formation in NH₄⁺-grown cells with and without ferricyanide, in the presence of 5% 1-propanol (representative experiments, n = 4). (A) Control, no [Fe(CN)₆]³⁻ (○), 1 mM [Fe(CN)₆]³⁻ added after 10 min (■). (B) Control, no [Fe(CN)₆]³⁻ (○), 1 mM [Fe(CN)₆]³⁻ present from the beginning (●).
Table II. Effect of KNO₃ on extracellular ferricyanide reduction in *Monoraphidium braunii*.

<table>
<thead>
<tr>
<th>KNO₃ (mM)</th>
<th>0</th>
<th>0.1–5</th>
<th>10–100</th>
<th>≥ 200</th>
</tr>
</thead>
<tbody>
<tr>
<td>% activity</td>
<td>100</td>
<td>107</td>
<td>84</td>
<td>60</td>
</tr>
<tr>
<td>n</td>
<td>12</td>
<td>9</td>
<td>12</td>
<td>6</td>
</tr>
</tbody>
</table>

was detected between 10 and 50 mM, greater nitrate inhibition only at very high KNO₃ concentrations beyond 100 mM, which means an unspecific salt effect.

**Discussion**

*Monoraphidium braunii* shows rates of ferricyanide reduction that are in a similar order of magnitude as with other plant material and algae if calculated on an hourly and dry weight basis (for *Monoraphidium* 1 mg chl = 24 mg dry wt.). With Fe-starved algae the rates are even in the upper range of the reported data [8,9] (10–56 μmol mg⁻¹ chl h⁻¹ = 7–39 μmol g⁻¹ dry wt. min⁻¹). Glucose did not stimulate ferricyanide reduction, even in the dark (Table I), although it is easily taken up and can substantially stimulate nitrate assimilation under some conditions [23]. The simultaneous acidification in the medium can be almost completely explained by the generation of new anionic charges by the reduction from (Fe(CN)₆)⁶⁻ to (Fe(CN)₆)⁴⁻ and charge equilibration by the weak cation, H⁺ [24]. The small excess in extracellular acidification is most easily explained by some uptake of K⁺, an ion available by the use of 2 mM K₃[Fe(CN)₆].

The question of the physiological or ecological role of extracellular reduction at such high rates has not been clearly answered yet. The hypothesis by Jones and Morel [19] assumes a role of the plasmalemma redox chain in nitrate reduction, nitrate uptake, and in reduction of other substrates, while other authors propose a function in stabilizing reduced membrane components and protection against oxygen radicals [25].

While the bulk of nitrate reductase in higher plants and algae has been found in the cytosol or attached to inner membranes, part of the enzyme has been localized in the plasmalemma in various organisms as in *Chlorella, Thalassiosira*, maize and barley roots. In *Neurospora* it was found to be attached to the tonoplast [26], in some unicellular green algae within the chloroplasts and associated to the pyrenoids [27].

The main evidence for the location in the plasmalemma has come from immunological studies, has also the evidence for its function in nitrate uptake at the plasmalemma [15–17]: NR antibodies were able to inhibit nitrate uptake as well as NR. In *Thalassiosira* they even inhibited extracellular reduction of Cu²⁺-bathophenanthroline disulfonate.

In immunological localization some problems might be involved, e.g. cross-reactions of polyclonal NR-antibodies with different proteins. This could be related to the complex structure of NR which shares some of its prosthetic groups and some partial activities with other enzymes. NAD(P)H dehydrogenase activity and flavoprotein are common to various enzymes in the cell, and even the molybdenum cofactor is very similar to that of some oxidases [28].

Jones and Morel [19] proposed that the NR located in the plasmalemma reduces intracellular nitrate, extracellular electron acceptors and also acts as a trans-plasmalemma proton pump. This assumption implies competition for electrons from NAD(P)H because both activities (reduction of nitrate and of extracellular acceptors) must share the initial part of the redox chain. Such a competition could only be prevented by supplying electrons to the terminal NR activity. Hence a mutual inhibition as found for Cu²⁺-bathophenanthroline disulfonate in *Thalassiosira* was also expected for ferricyanide and nitrate reduction in *Monoraphidium*. However, our results (Figs. 4 and 5) do not show this competition, indicating that the two activities are due to independent systems. Moreover, NAD(P)H production rates and NAD(P)H/NAD(P) ratios must be high enough to completely satisfy both activities, at least under our experimental conditions.

But there are also differences between the experiments with *Thalassiosira* and those reported here, in the electron acceptor as well as in the assay of nitrate reduction, for which Jones and Morel
used the production of primary amines and time intervals of up to 24 h. Production of primary amines includes the entire nitrate assimilation pathway, i.e., in addition to nitrate reductase, nitrite reductase and GOGAT need reducing equivalents. In the experiments of the present paper, nitrate reduction only was used to see the competition with extracellular ferricyanide reduction.

Quite different redox activities have been reported for the plasmalemma redox system \[8,9\]. The experiments of the present study use a redox activity that accepts electrons from an intracellular source and is stimulated by, but does not require, Fe-starvation. According to the literature \[8\], the plasmalemma redox system shows little specificity with respect to electron acceptors: ferricyanide, Fe\(^{3+}\)-EDTA, cytochrome c, O\(_2\), ferrisiderophores, dichlorophenol indophenol, Cu\(^{2+}\)-bathophenanthroline disulfonate etc., are easily reduced at the plasmalemma.

According to Jones et al. \[10\] the redox system in the plasmalemma of diatoms has a mid-point redox potential between \(-100 \text{ mV}\) and \(+94 \text{ mV}\). Crane and Barr \[8\] reported that compounds with a redox potential as low as 0 mV are able to serve as electron acceptors at the membrane surface of carrot cells. Since the redox potential of the couple \(\text{NO}_3^-/\text{NO}_2^-\) is \(+400 \text{ mV}\) \[29\], nitrate reduction would be thermodynamically possible for this unspecific system. However, in recent experiments \[30\] a purified plasma membrane-associated electron transport protein from maize roots was able to accept electrons from NAD(P)H to reduce ferricyanide and other acceptors but not at all nitrate. It may be questioned if these properties may allow for the low NR activity detected in plasmalemma fractions of \textit{Chlorella} and barley roots \[16,31\]. In \textit{Monoraphidium} 5—8\% of total NR activity must be accounted for. But even if it were so, addition of nitrate should inhibit extracellular ferricyanide reduction, or vice versa, by competition. The results of this study show very little inhibition in the presence of up to 50 mM KNO\(_3\), greater inhibition only at very high, unphysiological concentrations of \(\text{NO}_3^-\) and K\(^+\). The increased nitrate reducing activity of ammonium-grown algae in the presence of ferricyanide can be explained by an increased supply of reducing equivalents from the tricarboxylic acid or oxidative pentose phosphate cycles \[24\]. But apparently the electrons have an easier access to NR than to the extracellular ferricyanide reduction site.

From these data it can, at least, be excluded that the bulk enzyme of NR is responsible for the extracellular reduction of ferric compounds at the plasmalemma. It cannot be completely excluded that the constitutive isoenzyme of NR as described by Tischner \[17\] which may have a very low NR activity compared with other reducing activities, may have its main function in extracellular reduction, but no competition, rather independence, could be observed in the present experiments. It is not easy to imagine that the same protein should, in addition, be responsible for nitrate transport, as a low activity, constitutive transport system. If there is a close relationship between constitutive NR and constitutive nitrate transport, extracellular reduction seems to be independent of this system in \textit{Monoraphidium}.

**Acknowledgements**

The work was supported by a grant from the Ministerio de Educación y Ciencia in Madrid, Spain (A.C.) and by the Deutsche Forschungsgemeinschaft, DFG (R.P. and W.R.U.). The authors wish to thank Mrs. Ingrid Schroder for valuable technical assistance.

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