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Immunological studies of ferredoxin-nitrite reductases and ferredoxin-glutamate synthases from photosynthetic organisms

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Abstract. Polyclonal antiserum specific for ferredoxin-nitrite reductase (EC 1.7.7.1) from the green alga *Chlamydomonas reinhardii* recognized the nitrite reductase from other green algae, but did not cross-react with the corresponding enzyme from different cyanobacteria or higher plant leaves. An analogous situation was also found for ferredoxin-glutamate synthase (EC 1.4.7.1), using its specific antiserum. Besides, the antibodies raised against *C. reinhardii* ferredoxin-glutamate synthase were able to inactivate the ferredoxin-dependent activity of nitrite reductase from green algae.

These results suggest that there exist similar domains in ferredoxin-nitrite reductases and ferredoxin-glutamate synthases from green algae. In addition, both types of enzymes share common antigenic determinants, probably located at the ferredoxin-binding domain. In spite of their physicochemical resemblances, no apparent antigenic correlation exists between the corresponding enzymes from green algae and those from higher plant leaves or cyanobacteria.

Key words: Photosynthetic organisms – *Chlamydomonas reinhardii* – Ferredoxin-glutamate synthase – Ferredoxin-nitrite reductase – Common antigenic determinants – Ferredoxin-binding domain

Ferredoxin-nitrite reductase (Fd-NiR, EC 1.7.7.1) and ferredoxin-glutamate synthase (Fd-GOGAT, EC 1.4.7.1) are key enzymes for the photosynthetic assimilation of nitrate in higher plants, algae and cyanobacteria. Reduced ferredoxin serves as electron donor for the reactions catalyzed by these enzymes (Wallsgrove and Lea 1985).

Fd-NiR from higher plants and algae are markedly similar in their physicochemical and catalytic properties, and they contain 1 siroheme and 1 |4Fe-4S| cluster per enzyme molecule as prosthetic groups (Vega et al. 1980). Fd-NiR from *Anabaena sp.* 7119 has been purified as a single polypeptide chain of 52,000 Da (Méndez and Vega 1981). On the other hand, Fd-GOGAT from *Chlamydomonas reinhardii* (Márquez et al. 1986), spinach leaves (Hirasawa and Tamura 1984) and barley leaves (Márquez et al. 1988) have been purified until electrophoretic homogeneity, as a single polypeptide chain of M_r 140,000 to 170,000, containing flavin and iron-sulfur cluster as prosthetic groups.

Recently, specific polyclonal antisera were raised against purified preparations of Fd-NiR and Fd-GOGAT from *C. reinhardii.* The immunochemical characterization of both enzymes showed antigenic similarity at the corresponding ferredoxin-binding domain (Romero et al. 1988). In this work these antisera are used to explore the immunohistochemical properties of Fd-NiR and Fd-GOGAT from different higher plants, algae and cyanobacteria.

Materials and methods

Chemicals

The biochemical reagents for enzyme assays were from Sigma (St. Louis, USA). SDS-polyacrylamide gel electrophoresis materials were supplied by Bio-Rad (Richmond, USA). All other reagents were from Merck (Darmstadt, FRG).

Organisms and culture conditions

The green algae were grown at 25° C in liquid medium containing 10 mM KNO_3 as nitrogen source. *Chlamydomonas reinhardii,* strain 21 gr, used the medium previously described (Sueoka et al. 1967). *Monoraphidium braunii*, strain 202-7c, *Chlorella fusca,* strain 211-15, and *Chlorogonium sp.* were grown in the medium described by Kessler et al. (1963). The cyanobacteria species used were: *Anabaena variabilis*, strain PCC7942, *Nostoc sp.*, strain ATCC29105, and *Synechococcus*, strain PCC7942. These organisms were grown at 35° C in liquid medium as previously described (Herrero et al. 1981) with 20 mM KNO_3 as unique nitrogen source.

The culture medium for eukaryotic algae or cyanobacteria was maintained under continuous illumination with white fluorescent light (50 $W \cdot m^{-2}$), and was flushed with air supplemented with 5% (v/v) CO_2.

Field bean, pea, spinach, wheat and barley leaves were obtained from Instituto de Recursos Naturales y Agrobiología, C.S.I.C. (Sevilla, Spain).

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Abbreviations: Fd, ferredoxin; GOGAT, glutamate synthase; $MV^{\cdot+}$, reduced methyl viologen (radical cation); NiR, nitrite reductase; PMSF, phenylmethylsulphonyl fluoride; SDS, sodium dodecyl sulfate

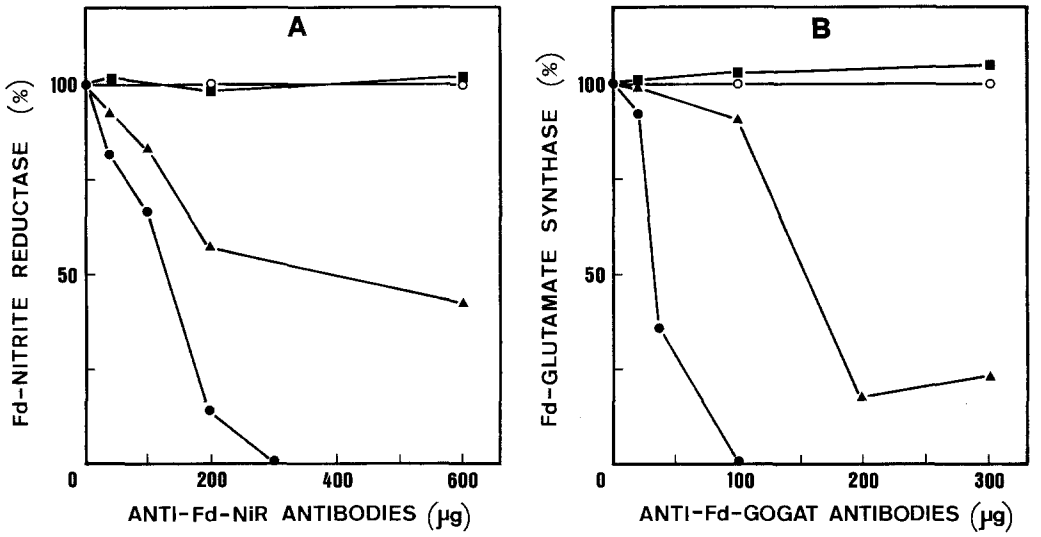

Fig. 1 A, B. Immunotitration curves of Fd-nitrite reductase and Fd-glutamate synthase from different photosynthetic organisms. Fd-NiR (2.5 nkat) or Fd-GOGAT (1.7 nkat) activities of crude extracts from *Chlamydomonas reinhardii* (●), *Monoraphidium braunii* (▲), and barley leaves (■) were incubated respectively with increasing concentrations of the antibodies raised against Fd-NiR and Fd-GOGAT from *C. reinhardii* to determine the level of remaining activity after the treatment. Open symbols are the respective controls with non-immune antisera. Other experimental details as described in Materials and methods

Preparation of crude extracts

C. reinhardii cells were broken by freezing in liquid nitrogen for 2 min and thawing in 10 mM K-phosphate buffer, pH 7.0, containing 14 mM 2-mercaptoethanol (5 ml · g^{-1} fresh weight). *M. braunii*, *C. fusca* or *Chlorogonium sp.* cells were broken by grinding them in a mortar with alumina powder (3 g · g^{-1} fresh weight) and the disrupted cell material was resuspended in the same buffer (5 ml · g^{-1} fresh weight). The resulting suspensions were centrifuged at 27,000 × *g* for 15 min, and the supernatants used as the corresponding crude extracts.

Cyanobacteria cells were resuspended in 50 mM K-phosphate buffer, pH 7.0, containing 14 mM 2-mercaptoethanol (5 ml · g^{-1} fresh weight), and broken by sonication at 80 W during 10 periods of 30 s each. The resulting homogenate was centrifuged at 27,000 × *g* for 15 min and the supernatant used for Fd-NiR studies. The crude extract for Fd-GOGAT activity was obtained by a similar procedure but using for extraction 50 mM K-phosphate buffer, pH 7.0, containing 28 mM 2-mercaptoethanol, 2 mM 2-oxoglutarate, 0.5 mM EDTA and 1 mM PMSF.

Green leaves from higher plants were cut in small pieces and ground in a mortar with glass beads of 0.1 mm diameter. The broken material was suspended (5 ml · g^{-1}) in 50 mM K-phosphate buffer, pH 7.5, containing 100 mM KCl, 2 mM 2-oxoglutarate, 5 mM EDTA, 14 mM 2-mercaptoethanol, 20% (v/v) ethylene glycol and 1 mM PMSF. The homogenate was filtered through 2 layers of gauze and centrifuged at 27,000 × *g* for 15 min. The supernatant was used as crude extract.

In all cases, the preparation of crude extracts was performed at 4° C.

Enzyme assays

The Fd-NiR activity was determined colorimetrically at 40° C measuring the nitrite consumed in the reaction mixture, which contained in 1 ml final volume: 150 µmol of Tris-HCl, pH 8.0; 2 µmol $NaNO_2$; an aliquot of the enzyme solution (1.7–3.3 nkat); and the electron donor system: 0.15 mg of *C. reinhardii* Fd or 2 µmol of methyl viologen, plus 10 µmol of sodium dithionite freshly dissolved in 0.3 M $NaHCO_3$ solution (Romero et al. 1987).

The Fd-GOGAT activity was determined colorimetrically at 30° C by measuring the glutamate formed in the reaction mixture, which contained in 2 ml final volume: 200 µmol of K-phosphate buffer, pH 7.0; 10 µmol of 2-oxoglutarate; 10 µmol of L-glutamine; 10 µmol of aminooxyacetate (transaminase inhibitor); an aliquot of the enzyme solution (less than 6.7 nkat); and the electron donor system: 0.15 mg of Fd or 20 µmol of methyl viologen, plus 23 µmol of sodium dithionite (Márquez et al. 1986).

Immunotitration of enzyme activities

Antibodies were obtained against purified preparations of Fd-GOGAT or Fd-NiR as previously described (Márquez et al. 1988; Romero et al. 1988). Constant amounts of either Fd-NiR or Fd-GOGAT activity were incubated at 4° C for 14 h with increasing concentrations of antibodies, in 0.5 and 1 ml final volume, respectively. After removal of the immunoprecipitates by centrifugation at 16,000 × *g* for 10 min, the corresponding enzyme activity was determined using aliquots of the supernatants.

Denaturing polyacrylamide gel electrophoresis of immunoprecipitates

The immunoprecipitates were collected by centrifugation at 16,000 × *g* for 10 min. Afterwards they were washed 3 times with 50 mM Tris-HCl, pH 7.5, containing 50 mM NaCl and 2% (v/v) Triton X-100, and twice with 10 mM Tris-HCl, pH 7.5. The washed immunoprecipitates were then dissolved by heating at 100° C for 5 min in 60 mM Tris-HCl, pH 6.8, containing 2% (w/v) SDS, 10% (v/v) 2-mercaptoethanol, 10% (w/v) sucrose and 20% (v/v) of a saturated Bromo-

Table 1. Immunoinhibition of Fd-nitrite reductase and Fd-glutamate synthase activities extracted from different photosynthetic organisms. Crude extracts from the indicated organisms were incubated at 4°C for 14 h with 600 µg of anti-*C. reinhardii*-Fd-NiR or 200 µg of anti-*C. reinhardii*-Fd-GOGAT antibodies respectively. After centrifugation, the remaining enzyme activities were determined using aliquots of the corresponding supernatants. The 100% were: 1.9 nkat (Fd-NiR and Fd-GOGAT) and 2.2 nkat ($MV^{\cdot+}$-NiR and $MV^{\cdot+}$-GOGAT).

Source of enzyme	Enzyme activities (%)			
	Fd-NiR	$MV^{\cdot+}$-NiR	Fd-GOGAT	$MV^{\cdot+}$-GOGAT
Chlamydomonas reinhardii	0	0	0	0
Monoraphidium braunii	42	74	18	37
Chlorella fusca	57	38	29	50
Chlorogonium sp.	43	80	20	34
Anabaena variabilis	96	113	nd	105
Nostoc sp.	121	120	nd	102
Synechococcus sp.	133	114	nd	105
Vicia faba (field bean)	70	87	89	89
Pisum sativum (pea)	95	98	91	92
Spinacia oleracea (spinach)	70	105	123	92
Triticum sp. (wheat)	96	87	106	97
Hordeum vulgare (barley)	100	100	102	98

nd, not determined

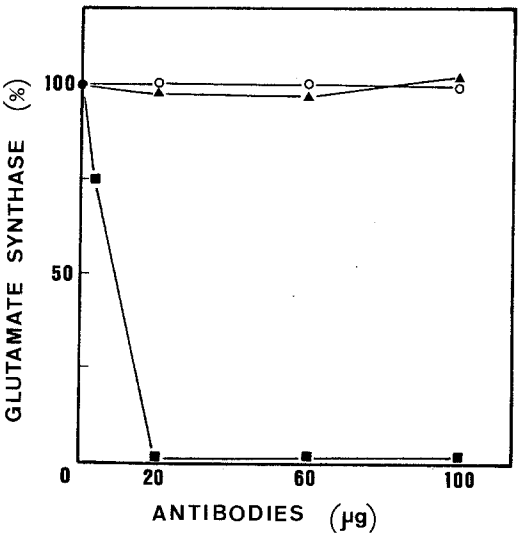

Fig. 2. Immunotitration curves of Fd-glutamate synthase from barley leaves and *Chlamydomonas*. Crude extracts from barley leaves (3.3 nkat, ■) or *C. reinhardii* (1.7 nkat, ▲) were incubated with increasing concentrations of anti-barley-Fd-GOGAT antibodies to determine the remaining level of Fd-GOGAT activity after the treatment. Open circles show the respective controls with non-immune antisera. Other experimental details as described in Materials and methods

phenol blue solution, with periodic shaking. After dissociation, the samples were run on SDS-polyacrylamide slab gels as described by Laemmli (1970), with 10% (w/v) and 4% (w/v) acrylamide concentrations for the separating and stacking gels, respectively. After electrophoresis the gels were stained with 0.1% (w/v) Coomassie blue R-250.

Results

Immunological comparison of Fd-nitrite reductases and Fd-glutamate synthases from photosynthetic organisms

Antisera raised against purified Fd-NiR or Fd-GOGAT from *Chlamydomonas reinhardii* were examined for its ability to immunoprecipitate respectively the Fd-NiR or Fd-GOGAT from different photosynthetic organisms (Fig. 1). 300 µg of anti-Fd-NiR antibodies are necessary to fully inhibit 2.5 nkat of *C. reinhardii* Fd-NiR activity. However, at this antibody concentration, only 50% of the Fd-NiR from *Monoraphidium braunii* was inhibited, whereas no effect was observed on the enzyme from a barley leaf extract (Fig. 1 A). An analogous situation was found when the Fd-GOGAT was studied (Fig. 1 B). Meanwhile 100 µg of specific antibodies were sufficient to inhibit completely the Fd-GOGAT from *C. reinhardii*, 3-fold higher amount of antibodies only produced a 75% inhibition on the enzyme from *M. braunii*, and did not affect the barley Fd-GOGAT.

Since antigenic differences were observed between the enzymes studied, the immunological comparison was extended to enzymes from other green algae, higher plants or cyanobacteria. Table 1 summarizes the results obtained in the screening. Fd-NiR and Fd-GOGAT from other green algae, such as *Chlorella fusca* or *Chlorogonium sp.* are also strongly inhibited when incubated with the corresponding antibodies. No specific immunoprecipitates were detected by SDS electrophoresis or Ouchterlony double immunodiffusion, excepting for the reactions of *C. reinhardii* Fd-NiR or Fd-GOGAT with their specific antibodies. This indicates that the observed cross-inhibition was not due to immunoprecipitation of the antigen-antibody complexes, but rather probably to specific immunocomplexes formed is solution.

No effect of *C. reinhardii* antibodies was detected on the Fd-NiR or Fd-GOGAT from fixing or non fixing cyanobacteria such as *Anabaena variabilis*, *Nostoc sp.* or *Synechococcus sp.* (see Table 1). With regard to higher plants, the maximal inhibition was 30%, and only for Fd-NiR activity from some dicotyledonous species (field bean and spinach). Consequently, no evidence for a significant degree of cross-reactivity can be established between *C. reinhardii* Fd-NiR and Fd-GOGAT antibodies and the corresponding enzyme from higher plant leaves.

Studies of cross-reactivity between Fd-glutamate synthase from Chlamydomonas and barley leaves

While the Fd-GOGAT from barley was efficiently inhibited by an antiserum raised against the purified Fd-GOGAT from barley leaves (Fig. 2) no effect was observed on the corresponding enzyme from *C. reinhardii*, thus confirming the lack of cross-reactivity indicated by the use of antibodies raised against the enzyme from *C. reinhardii* (see Fig. 1). Figure 3 shows the results of SDS-polyacrylamide gel electrophoresis of the immunoprecipitates formed in the reactions between barley and *C. reinhardii* Fd-GOGAT containing extracts with their respective antibodies. Besides the IgG chains, an unique protein band of M_r about 150,000, corresponding to the Fd-GOGAT appeared in the gel, thus indicating the high specificity of the used antibodies. This

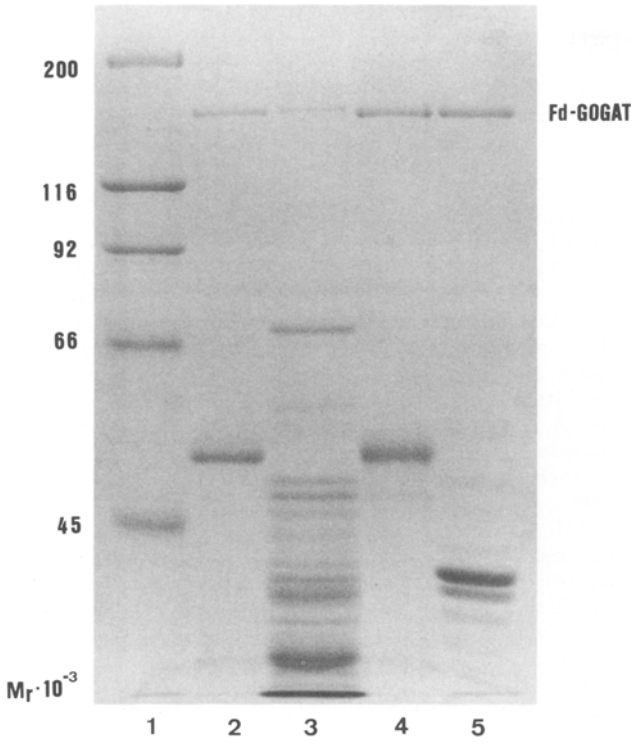

Fig. 3. SDS-polyacrylamide gel electrophoresis of the immunoprecipitated Fd-glutamate synthase from barley leaves and *Chlamydomonas*. *Lane 1* Molecular weight markers. *Lane 2* Immunoprecipitate from barley extract using anti-barley-Fd-GOGAT antibodies. *Lane 3* Crude extract from barley leaves. *Lane 4* Immunoprecipitate from *C. reinhardii* extract using anti-*C. reinhardii*-Fd-GOGAT antibodies. *Lane 5* Partially purified extract from *C. reinhardii*

Table 2. Immunoinhibition of nitrite reductase from green algae using anti-*Chlamydomonas*-Fd-GOGAT antibodies. Crude extracts from the indicated green algae were incubated at 4° C during 14 h with 600 μg of antibodies raised against Fd-GOGAT from *C. reinhardii*. After centrifugation, methyl viologen and ferredoxin-dependent NiR activities were determined and expressed as percent of the initial values (1.9 nkat for Fd-NiR and 2.2 nkat for $MV^{\bullet+}$-NiR)

Source of Enzyme	Nitrite reductase activity (%)	
	Fd-NiR	$MV^{\bullet+}$-NiR
Chlamydomonas reinhardii	0	125
Monoraphidium braunii	55	109
Chlorella fusca	69	116
Chlorogonium sp.	33	110

experiment also shows that, independently of the lack of cross-reactivity, the Fd-GOGAT from barley leaves and *C. reinhardii* have similar electrophoretic mobility, and thus, molecular weight.

Studies of cross-reactivity between Fd-nitrite reductase and Fd-glutamate synthase from green algae

It has been also examined the possibility that anti-*C. reinhardii*-Fd-GOGAT antibodies could recognize the Fd-NiR from other green algae, as it was shown previously for the Fd-NiR from *C. reinhardii* (Romero et al. 1988). Table 2 indicates that a strong inhibition of the Fd-NiR from *M. braunii*, *C. fusca* and *Chlorogonium sp.* is produced by the mentioned antibodies. However, this effect is observed only on the Fd-dependent activity, but not when $MV^{\bullet+}$ was used instead of reduced Fd as electron donor.

Discussion

The results reported here indicate the presence of common antigenic determinants in the Fd-NiR from green algae, which are not conserved in the corresponding enzyme from other photosynthetic organisms. These antigenic differences contrast with the similarities found in their physicochemical properties, kinetic parameters and prosthetic groups composition (Vega et al. 1980). An analogous situation was also shown for the Fd-GOGAT in spite of the resemblances found between the enzymes purified from spinach, barley and *Chlamydomonas reinhardii* (Hirasawa and Tamura 1984; Márquez et al. 1986, 1988). In fact, identical electrophoretic mobilities on SDS-gels were shown for the immunoprecipitated Fd-GOGAT from barley leaves and *C. reinhardii* (Fig. 3).

However, it is interesting to emphasize that the Fd-NiR and Fd-GOGAT seem to share common antigenic determinants which are conserved in different green algae. A previous work done in *C. reinhardii* showed that these antigenic determinants could be located at the corresponding ferredoxin-binding domains (Romero et al. 1988). This was basically supported by two facts: 1. The anti-Fd-GOGAT antibodies specifically immunoprecipitate the Fd-dependent activity of the Fd-NiR but not that dependent on $MV^{\bullet+}$. 2. The addition of purified ferredoxin to the antigen-antibody mixture prevents the inactivation of the Fd-NiR. The results reported in Table 2 are consistent with this observation.

Immunological comparisons of the Fd-NiR and Fd-GOGAT have only been made in higher plants. It has been reported the existence of common antigenic determinants in the Fd-NiR from green and non-green tissues of spinach and bean (Hirasawa et al. 1984; Ishiyama et al. 1985) and also between the Fd-NiR from dicotyledonous plants (Ida 1987). On the other hand, antibodies raised against the Fd-GOGAT from rice leaves partially recognized the enzyme from root tissues of this plant (Suzuki et al. 1982), and showed cross-reaction with the Fd-GOGAT from tobacco cultured cells (Suzuki et al. 1984a) and the plant cell fraction of soybean nodules (Suzuki et al. 1984b). Curiously the antigenic homology found in the Fd-NiR or Fd-GOGAT from higher plants is different from that found in green algae since no cross-reaction was observed between both groups of organisms.

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