Phytochemistry, 1978, Vol 17, pp. 35–39. Pergamon Press. Printed in England

ISOLATION AND PROPERTIES OF CRYSTALLINE FERREDOXIN FROM *LACTUCA SATIVA*

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(*Revised received* 8 *July* 1977)

Key Word Index—*Lactuca sativa*; Compositae; lettuce; purification; phylogeny, ferredoxin; fructose-1,6-diphosphatase.

Abstract—Lettuce ferredoxin has been purified to homogeneity, with a yield of 18 mg/kg of denerved leaves. It crystallizes in magnificent needles, often clustered in broom-like sheaves. The absorption spectrum showed maxima at 460, 422, 330 and 274 nm,with a ratio A_{422}/A_{274} of 0.46. The mM absorption coefficient was 9.74 at 422 nm, and 21.62 at 274 nm. This ferredoxin showed a pI = 4.7 and an $E_0' = -425$ mV (at pH = 7.7). MWs of 12400, 11480 and 13000 were obtained by sucrose gradient centrifugation, and on the basis of the amino acid composition and the iron content, respectively, with an average of 12300. The amino acid analysis showed the existence of one methionine residue per mole, with 105 amino acid residues. There are two iron atoms and two labile sulfide groups per mole; 4 half-cystine residues were found by performic acid oxidation, and 5 cysteine groups when determined by titration with *p*HMB. The native protein is not fixed on thiol-Sepharose 4B, but it is quantitatively retained after incubation with 8 M urea. Lettuce ferredoxin showed a 62, 58 and 78 % effectiveness with the spinach ferredoxin-NADP reductase, nitrite reductase and fructose-1,6-diphosphatase (FDPase), respectively, when compared with the spinach ferredoxin. This different behaviour of both ferredoxins is joined to genetic–structural relationships, and suggests that the role of ferredoxin in FDPase activation is more sophisticated than that of a mere nonspecific reductant.

INTRODUCTION

The occurrence in spinach leaves of a protein factor catalyzing the reduction of methemoglobin by illuminated chloroplasts was first described by Davenport *et al.* [1]. Later, Tagawa and Arnon [2] coined the term ferredoxin for this type of substances. Since then, new aspects concerning these compounds have been discovered, and discussed in some review articles [3–5].

Concerning algal and plant ferredoxins their phylogenetic relationships become complicated because all of them seem to hold similar active centres [6] and, so far, only ferredoxins from two *Spirulina* species [7, 8], *Aphanotece sacrum* [9] and *Nostoc muscorum* [10] (prokaryote blue-green algae), *Scenedesmus* [11] (green alga), *Colocasia esculenta* [12] (monocotyledonae), and spinach [13], alfalfa [14] and *Leucaena glauca* [15] (dicotyledonae) have been sequenced. Partial sequential studies have been also made on ferredoxins from the red alga *Porphyra umbilicalis* [16], the fern *Equisetum talmateia* [17] and the dicotyledonous *Sambucus* [18]. Noteworthy differences have been found in the aminoacid composition or primary structure of some of these groups, and the quantitative changes of effectiveness observed in some ferredoxin-dependent reactions can be imputed to them.

This work tries to overcome the lack of knowledge on ferredoxins from Compositae that, as the most evolved family of dicotyledonous Angiosperms, may be useful for the elucidation of phylogenetic problems. The physico-chemical properties of lettuce ferredoxin and its specific involvement in some enzymatic reactions are studied, emphasizing the photosynthetic FDPase, whose ferredoxin-dependence was discovered by Buchanan *et al.* [19], and which is being studied in our laboratory [20, 21].

RESULTS AND DISCUSSION

Ferredoxins have been purified from different sources. Apart from bacteria and algal ferredoxins, we must mention among the ferns those from *Equisetum* [22] and *Polystichum* [23]; and those from *Pinus pinea* [24], nutsedge [25], *Colocasia esculenta* [26], corn [27], spinach [2], *Amaranthus* [23], parsley [28], cotton [29], *Leucaena glauna* [15], alfalfa [30], pea [31], soybean [32], *Chenopodium* [33], *Sambucus* [18], watermelon [34] and squash [35] among the higher plants.

The purification procedure of lettuce ferredoxin is summarized in Table 1, with a yield of 4 mg of pure ferredoxin per kg of rough leaves, that becomes 18 mg/kg when referred to depetioled and denerved green leaves of market lettuces. The purity of lyophilized protein is shown by the high value of the A_{422}/A_{274} ratio; after acrylamide gel electrophoresis only one band appears, either with amidoblack or non-heme iron staining.

With the exception of the large hexagonal plates

Table 1. Purification of ferredoxin from lettuce leaves

Purification step	Volume (ml)	Protein (mg)	A_{422}/A_{274}	Purification (×)
Crude extract	2300	20800	—	1 0
Acetone precipitation	170	1300	—	16.2
Adsorption on DEAE-cellulose	40	356	0 17	58.5
Chromatography on DEAE-cellulose	140	103	0 33	201
Filtration through Sephadex G-75	43	32	0 46	650

Starting material: 8 kg of leaves. Yield: 4 mg/kg of rough leaves, corresponding to 18 mg/kg of denerved green leaves.

prepared from *Chenopodium album* [33], ferredoxins from higher plants show only weak crystallization, and only those from spinach [36], parsley [37] and squash [35] have been so far obtained as minute crystals. On the contrary, good results have been found with the proteins from the algae *Nostoc* [38], *Euglena* [39] and *Phormidium* [40] and from different clostridial species [41]. We have got from lettuce ferredoxin magnificent red needles, often clustered in broom-like sheaves (Fig. 1), very probably because of the slow increase in $(NH_4)_2SO_4$ concentration by dialysis; its crystalline condition was checked by visualization with polarized light.

The spectral properties of lettuce ferredoxin are similar to those of other ferredoxins from higher plants, with maxima at 460, 422, 330 and 274 nm, and relative absorbances of 0.41, 0.46, 0.66 and 1, respectively. Like spinach protein [36], the absorption peak at 422 nm decreases on reduction to 50% of the original value, whereas the reduction by a high excess of sodium dithionite was followed by total bleaching and disappearance of the 460 and 422 nm peaks. The mM extinction coefficient was 9.74 at 422 nm, and 21.6 at 274 nm. The pI of ferredoxins has only been determined in a small number of cases because of their known instability to acidic pHs. Using starch gel electrophoresis Lovenberg *et al.* [41] found a pI of 3.7 in clostridial ferredoxin, lower than the value 4.7 that we have determined for the lettuce ferredoxin by electrofocusing in sucrose gradient; this can be explained because of the almost complete lack of basic aminoacids in the former. An intermediate value of 4 was found in *Chenopodium* ferredoxin using electrofocusing in acrylamide gel [33]. The oxidation–reduction potential was determined in duplicate experiments, with two equilibrium conditions each; results are shown in Table 2, with an average value of −0.425 V. This value is close to that of −420 mV found in spinach [36], supporting the identity of chloroplastic ferredoxins concerning their reducing power and electron transfer properties.

Table 2. Oxidation-reduction potential of lettuce ferredoxin

	$[MV_{red}]$ (%)	$[Ferredoxin_{red}]$ (%)	$\frac{[MV_{ox}][Ferredoxin_{red}]}{[MV_{red}][Ferredoxin_{ox}]}$	E_0 (volts)
Expt I	71.4	88.9	3.20	−0.417
	65.7	83.4	2.63	−0.421
Expt II	70.0	79.2	1.64	−0.433
	48.1	64.9	2.00	−0.428

Lyophilized ferredoxin was dissolved at *ca* 0.1 mM concn in 1.5 ml of 0.05 M Tris–HCl buffer pH 7.7, made up 0.1 mM in MV. The mixture was anaerobically reduced with 15 µl of 100 mN Na dithionite, and then stepwise reoxidized by admission of *ca* 24 µl aliquots of air. After 10 min equilibrium the *A* at 740 and 422 nm were measured.

On the basis of the MW obtained by centrifugation, 1.91 atoms of Fe and 1.86 labile sulfide groups were calculated per mole of protein. So, the lettuce ferredoxin appears to have two atoms each of non-heme Fe and acid-labile S per mole, as usual in chloroplast ferredoxins. Sucrose gradient centrifugation showed a MW of 12400 and a sedimentation coefficient of 1.74 S, when the ferredoxin behavior was compared with that of cytochrome *c*, standard with the nearest sedimentation position. A MW of 6490 per mole was obtained from the Fe content, and since parallel analysis gave two Fe atoms per mole, a MW of 13000 was established. The amino acid composition, as a mean of 3 analyses, is shown in Table 3. With leucine normalized to 8 residues per mole, and after nearest integration, we have obtained 105 residues per mole and a MW of 11300 for the polypeptidic chain of the protein, that when supplemented with two Fe and two labile sulfide gave a final value of 11480. For purposes

Fig. 1. Photomicrograph of crystalline lettuce ferredoxin (× 1250).

Table 3. Amino acid composition of lettuce ferredoxin, and comparison with other chloroplastidic ferredoxins

Amino acids	Residues per molecule					
	Lettuce*		*Euglena* [39]	*Equisetum* [22]	Corn [27]	Spinach [13]
	Normalized to 8 leucines	Nearest integer				
Lysine	5.42	5	5	4	3	4
Histidine	2 00	2	1	1	2	1
Arginine	2.00	2	1	1	1	1
Aspartic acid	14 00	14	14	8–9	13	13
Threonine	7 43	7	9	7	5	8
Serine	8 86	9	8	8	8	7
Glutamic acid	13 71	14	9	15–16	14	13
Proline	6 29	6	4	4	4	4
Glycine	8 86	9	7	9	8	6
Alanine	6 29	6	8	6	8	9
Valine	8 00	8	6	6	10	7
Isoleucine	4 29	4	4	5	5	4
Leucine	8 00	8	7	8	8	8
Phenylalanine	3.14	3	3	4	1	2
Tyrosine	3 12	3	1	2	5	4
Tryptophan	not determined		0	0	1	1
Methionine	0 86	1	0	1	0	0
½ Cystine	4 00	4	6	4	4	5
Total residues		105	93	93–95	100	97

*Average value of 3 analyses. Samples were hydrolyzed with 6N HCl in sealed tubes *in vacuo* for 20 and 70 hr at 110°. Serine and threonine were corrected by extrapolation to 0 time hydrolysis. Half-cystine was determined after performic acid oxidation.

of comparison we also include in this Table the amino acid composition of ferredoxins from other sources.

The MWs determined by these 3 methods gave a mean value of 12300, which is in agreement with those from other higher plant ferredoxins. The amino acid composition of lettuce ferredoxin shows similarities with other higher plant ferredoxins, but it differs from other plant ferredoxins in containing one methionine residue per mole, as also occurs in those from *Amaranthus* [23], nutsedge [25], cotton [29] and squash [35]. We have determined 105 amino acid residues per mole, higher than the 90–95 residues of algal and fern ferredoxins, and the 95–100 of those from other higher plant ferredoxins.

The end point of titration with *p*HMB corresponded to 8.75 equivalents of mercurial per mole of ferredoxin. If it is assumed that 4 moles of *p*HMB react with the 2 labile sulfide, the remaining 4.75 equivalents would be spent in the titration of non-labile —SH groups; accordingly, lettuce ferredoxin appears to have 5 cysteine residues per mole. Nevertheless, they differ from the 4 half-cystine groups obtained by performic acid oxidation. Without incubation with urea, native lettuce ferredoxin is not fixed at all, but it is retained quantitatively on the thiol-Sepharose after 2 hr incubation in the presence of the denaturant (Table 4).

Table 4. Availability of —SH residues of lettuce ferredoxin by covalent chromatography on thiol-Sepharose 4B

Material	A_{280}	A_{343}	A_{422}	A_{422}/A_{280}	A_{343}/A_{280}
Native ferredoxin					
Non fixed and washed material	0.65	0 44	0.30	0 46	0 68
Material eluted with 10 mM cysteine	>2	>2	0	0	ND
Ferredoxin incubated with 8M urea.					
Non fixed and washed material	0 52	0 51	0	0	1.02*
Material eluted with 10 mM cysteine	>2	>2	0 05	<0 025	ND

ND: not determined because of the high release of 2-thiopyridone after elution with cysteine. *A 2-thiopyridone soln does not absorb in the visible, and shows a A_{343}/A_{280} ratio of *ca* 1.

Table 5. Biological activity of lettuce ferredoxin in some ferredoxin-dependent systems from spinach

Ferredoxin-NADP reductase (μM of $NADP^+$ reduced/min/mg of chlorophyll)	Nitrite reductase (μM of NO_2^- reduced/min/ml of ferredoxin soln)	FDPase (μM of PO_4^{3-} released/min/ml of ferredoxin soln)
Spinach ferredoxin		
0 84	0.59	12 2
0 84	0 57	10 9
0 86	0 61	10 9
Mean 0 85	0 59	11 3
Lettuce ferredoxin		
0 50	0 36	8.2
0 52	0 34	8 8
0 56	0 33	9 3
Mean 0 53	0 34	8.8
Effectiveness (%) lettuce/spinach		
62	58	78

The availability of mercurial to the —SH groups and the non fixation of native ferredoxin to the Sepharose could be explained either as a stronger reactivity of the former with the —SH groups blocked in the active centre, or as a bigger volume of the dithiopyridinic residue of the Sepharose, assumed that the active group is buried in the structure of the molecule and there are steric obstacles to its availability. In any case, urea incubation not only breaks the protein structure making available the active centre if buried, but also destroys the structure of the active group, explaining the quantitative fixation on the Sepharose of urea treated ferredoxin.

Table 6 shows the effectiveness of spinach and lettuce ferredoxins in the 3 ferredoxin-dependent systems from spinach leaves tested, of which the earlier references in plants are only concerned with ferredoxin-NADP reductase system. Great differences of effectiveness between bacterial and plant ferredoxins were found in the NADP reduction by illuminated chloroplasts. On the contrary, algal and higher plant ferredoxins are very similar in efficiency, with the exception of that from the alga *Bumillieropsis filiformis* [42], which shows with spinach chloroplasts a lower activity than the homologous protein. The 62% effectiveness found with lettuce ferredoxin in comparison with spinach ferredoxin, is supported with a 58% operativity in the nitrite reductase test.

Buchanan *et al.* [19] found the *in vitro* activation of photosynthetic FDPase by reduced ferredoxin, that could be non-physiologically replaced by dithiothreitol. This effect was maximal at pH 7.9, close to that of the stroma of illuminated chloroplasts [43]; indeed, there is FDPase activity in the chloroplast only under illumination [44]. These results provided evidence that a reductant, which may well be reduced ferredoxin, is developed in the chloroplast under illumination, activating the FDPase by an unknown mechanism. The reductive formation of sulphydryl groups essential for enzyme activity has been a proposed explanation [44], supported by the large half-cystine content reported for the FDPase [19].

However, if a simple reduction mechanism takes place, it would be difficult to explain the lack of effectiveness of *Cl. pasteurianum* ferredoxin—the only heterologous ferredoxin earlier assayed—in spinach FDPase activation [19]. El-Badry [45] found that the mechanism of activation by reduced ferredoxin differs from that of dithiothreitol. More recently we have determined

16 half-cystine residues per mole of FDPase [21], in contrast with the 210 found by the Buchanan's group [19].

All these events, in addition to the 74 % effectiveness found for lettuce ferredoxin in comparison with the homologous protein from spinach, suggest that the role of ferredoxin in FDPase activation is more sophisticated than that of a mere nonspecific reductant. This is in agreement with the complex molecular structure we have found in photosynthetic FDPase [20, 21], its complicated mechanism of action [19] and important regulatory role in the Calvin cycle [46].

EXPERIMENTAL

Preparation of crude extracts of the whole leaves. Batches (8 kg) of fr. lettuce leaves, devoid of petioles and base of limb, were washed and dried between filter papers; prominent veins were removed and the laminae frozen at −20° in plastic bags. The leaves were crushed by hand and homogenized (4:3 w/v) in 0.1 M Tris–HCl buffer pH 7.2, 0.1 % Triton X-100, for 3 min at max. speed (Sorvall omni-mixer). After filtering through 3 layers of nylon cloth, the extract was centrifuged at 20000 *g* for 30 min at 4°.

Purification of ferredoxin. Protein content was determined according to ref. [47] and the purity tested by measuring the ratio A_{422}/A_{274}, λ_{max} of this ferredoxin. The homogeneity of the final prepn was checked by disc electrophoresis in polyacrylamide gel (15 % acrylamide), according to ref. [48]; gels were stained with amidoblack 10B, and with 2,2′-dipyridyl as detector of non-heme Fe [49]. The method described in ref. [2] for spinach ferredoxin was basically followed. The procedure consisted of the following steps: Me_2CO pptn, adsorption on DEAE-cellulose, followed by chromatography on DEAE-cellulose and concn using the same ion exchanger. The method was modified performing the chromatographic step on a DEAE-52 cellulose column (2.5 × 30 cm), equilibrated beforehand with 0.15 M Tris–HCl buffer pH 7.3, adjusted to 0.2 M in Cl^- by addition of NaCl, the elution of fixed material was carried out with a linear gradient established between 300 ml of equilibration buffer and a sufficient vol. of the same buffer made up 0.37 M in Cl^-. Some impurity traces were eliminated by later filtration through a Sephadex G-75 column (2.5 × 80 cm) equilibrated and afterwards eluted with 0.01 M Tris–HCl buffer pH 7.3, at a flow rate of 0.5 ml/min. Fractions (10 ml) with a A_{422}/A_{274} ratio of 0.46 were combined and dialyzed overnight against Tris–HCl buffer pH 7.3, and then lyophilized and stored under N_2 at −30°.

Crystallization. A conc ferredoxin soln was microdialyzed according to ref. [50] at 4°, for 24 hr, against 80, 90 and 95 % $(NH_4)_2SO_4$ satd soln. After 3 days a turbidity was observed, and crystals were collected by centrifugation at 10000 *g* for 15 min.

Spectral properties. The absorption spectrum of oxidized ferredoxin was recorded in the range 220–700 nm. Reduced ferredoxin was prepared by mixing anaerobically 1.5 ml of 0.1 mM ferredoxin soln with 10 µl of 100 mM Na dithionite in a Thunberg-type cell; an irreversible reduction was obtained with 25 µl of reductant. The ε value was determined at 422 and 274 nm with lyophilized ferredoxin dissolved in 5 mM Tris–HCl buffer pH 7.3. The calculation was made on the basis of a MW of 12300, determined as stated below.

pI was determined by electrofocusing in a sucrose density gradient (10–35 %), according to ref. [51]. Tubes of 0.8 × 15 cm were used, with ampholines (LKB) of pH range 3.5–10 at 2.1 % final concn. The tubes were eluted at 0.4 ml/min, 0.4 ml fractions collected, and their pH and 422 nm *A* were measured.

Oxidation–reduction potential. The E'_0 value was determined basically as described in ref. [52]. A mixture of *ca* equimolar amounts (0.1 mM) of Me viologen (MV) and ferredoxin were anaerobically reduced in a Thunberg cuvette with a slight excess of Na dithionite, and then reoxidized stepwise by repeated admission of small amounts of air. After 10 min of each admission the concn of the oxidized and reduced species of MV and ferredoxin were determined, on the assumption that the MV does not absorb in the visible in the oxidized form, whereas it shows a maximum at 740 nm in the reduced state, a λ in which the absorption by ferredoxin is negligible. The redox potential was calculated from the equation:

$$E'_0 = E_0 + \frac{RT}{F} \ln \frac{[MV_{ox}][Ferredoxin_{red}]}{[MV_{red}][Ferredoxin_{ox}]}$$

in which E_0 is the normal oxidation–reduction potential of MV system (−446 mV at pH 7.7 of the expt conditions).

MW was determined by density gradient centrifugation and on the basis of the amino acid composition and the Fe content. Samples of 0.1 ml were layered on a 2–10 % linear sucrose gradient established in 4.6 ml of 0.05 M Tris–HCl buffer pH 7.3, and centrifuged at 4° in the 6 × 5 ml 2414 swinging-bucket rotor of a MSE Superspeed 40 ultracentrifuge, for 14 hr at 130000 *g*. Cytochrome *c*, myoglobin and chymotrypsinogen A (Serva, Feinbiochemica) were used as standards in parallel runs. The gradients were drained upward at a flow rate of 0.4 ml/min beginning at the bottom, monitored at 280 nm through a continuous flow cell, and collected with a tube change every 20 sec (36 fractions with *ca* 0.13 ml each). They were tested for specific identification. According to ref. [53] the MW and sedimentation coefficient were obtained from from the equation:

$$\frac{A}{A'} = \frac{S_{20,w}}{S'_{20,w}} = \left(\frac{MW}{MW'}\right)$$

in which *A* and *A′* are the distances travelled from meniscus by the unknown and standard, respectively.

Amino acid composition. Ferredoxin was hydrolysed with 6 M HCl at 110° in sealed tubes *in vacuo* for 20 and 70 hr. The amino acids were determined in the 20 hr hydrolyzates with an autoanalyzer; the serine and threonine contents were corrected by extrapolation to zero time. Total half-cystine was determined as cysteic acid in a separate sample after performic acid oxidation. The MW of lettuce ferredoxin was calculated by normalization to different leucine contents, and supplemented by the Fe and labile sulfide groups.

Fe and labile sulfide. Fe was determined by atomic absorption spectrophotometry after nitric–perchloric digestion; MW was obtained from different molar Fe contents. Labile sulfide was determined colorimetrically according to ref. [54].

Determination and availability of —SH residues. The number of —SH groups of lettuce ferredoxin was determined by spectrophotometric titration with *p*HMB, as described in ref. [55], and their availability was tested by covalent chromatography on thiol-Sepharose 4B [56]. All the following experiments were performed aerobically. A ferredoxin soln in 0.1 M Tris–HCl buffer pH 7.3, 0.3 M in NaCl, was applied at a flow rate of 4 ml/min to a thiol-Sepharose 4B (Pharmacia, Uppsala) column (0.9 × 3 cm), equilibrated beforehand with the same buffer. In a parallel experiment the ferredoxin soln was previously incubated for 2 hr at 4° with 8 M urea, and then applied to another column equilibrated with the same buffer made up 8 M in urea. After washing both columns with 6 ml of buffer without urea, they were eluted with 10 mM cysteine in the washing soln. 1.5 ml fractions were collected and tested for proteins (λ = 280 nm), ferredoxin (λ = 422 nm) and the 2-thiopyridone (λ = 343 nm) released in the fixation of —SH compounds. Fractions of the same peak were mixed and their *A* at the above wavelengths again determined.

Biological activity in ferredoxin-dependent reactions. The tests were performed with spinach or lettuce ferredoxins at the same molar concn, that was determined by *A* at 422 nm and further correction for their molar absorption coefficient. Spinach ferredoxin, homogeneous on acrylamide gel electrophoresis, with a A_{420}/A_{274} ratio of 0.49 was prepared according

to ref. [2]. For nitrite reductase assay the reaction mixture was : 0.3 ml of 0.5 M Tris–HCl buffer pH 8, 0.3 ml of 2.5 % Na dithionite, 0.2 ml of ferredoxin soln, 0.25 units of spinach nitrite reductase, and 0.2 ml of 20 mM $NaNO_2$, in a final vol. of 2 ml. After 10 min incubation at 30° the remainder NO_2^- was determined as described in ref. [57]. Nitrite reductase from spinach leaves was obtained by the method of ref. [58]. The ferredoxin-NADP reductase test was carried out in a quartz cuvette containing 0.1 ml each of M Tris–HCl buffer pH 7.8, 2 mM 2,6-dichlorophenolindophenol, 0.2 M Na ascorbate, 10 mM $NADP^+$, washed spinach chloroplasts equivalent to 0.05 mg of chlorophyll, and ferredoxin soln. in a final vol. of 3 ml. The reaction was started by illumination of 10 klx, and the reduced NADPH determined by the increase in A at 340 nm, every 3 min. Washed spinach chloroplasts were the C_1s_2 particles prepared according to ref. [59]; chlorophyll content was determined by the method of ref. [60]. For FDPase assay the following was added to the main body of a Thunberg-type cell: 0.1 ml of 0.66 M Tris–HCl buffer pH 8, 25 µg of spinach FDPase, 0.2 mg of spinach protein factor, and 50 µl each of 1.32 mM Na EDTA, 20 mM $MgCl_2$, 1.3 mM 2,6-dichlorophenolindophenol, 133 mM Na ascorbate, 70 mM reduced glutathion, and ferredoxin soln, in a final vol. of 0.9 ml; and in the side arm 50 µl each of 80 mM fructose-1,6-diphosphate, and washed spinach chloroplasts equivalent to 0.065 mg of chlorophyll. After 15 µm vacuum the reaction was started by mixing both compartments, and carried out under illumination of 10 klx, for 30 min at 20°. The released Pi was determined according to ref. [61]. FDPase was obtained from spinach leaves by the method desribed in ref. [62], and the protein factor according to ref. [19]. Washed spinach chloroplasts are the same particles stated above.

Acknowledgements—This research was supported by financial assistance from the Comisión Asesora de Investigación Científica y Técnica (Spain). We are grateful to Miss Matilde Garrido for technical assistance.

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