Biochimica et Biophysica Acta, 396 (1975) 293–300

BBA 46965

IMMUNOLOGICAL QUANTITATION OF CHLOROPLAST FERREDOXIN

ROBERT S. MATSON and TOKUJI KIMURA

Department of Chemistry, Wayne State University, Detroit, Mich. 48202 (U.S.A.)

(Received December 9th, 1974)
(Revised manuscript received April 29th, 1975)

SUMMARY

An immunoassay for the quantitative determination of ferredoxins in cell-free extracts from plant tissues is described. The method is accurate for the assay of 0.3–1.5 nmol ferredoxin directly from the extracts. The following average values (nmol ferredoxin/mg extractable protein) were obtained: 3.9, 1.8, 5.90, 14.8 and 10.9 for *Euglena gracilis*, spinach, parsley, lettuce and broccoli, respectively. Specific factors affecting the method are discussed in detail.

INTRODUCTION

The content of ferredoxin in biological materials has been determined by various methods [1–3]. Such methods lack strict specificity for the measurement of ferredoxin. We wish to report here the quantitative determination of plant ferredoxins based upon an immunological method employing the antibody to spinach ferredoxin and its inhibitory effect on the NADPH-flavoprotein-mediated reduction of cytochrome *c*. A reliable measure of the ferredoxin content in various green plants and the photosynthetic protist, *Euglena gracilis* was obtained due to their cross-reactivity with spinach ferredoxin.

MATERIALS AND METHODS

NADPH (type II) and cytochrome *c* (horse heart, type III) were purchased from Sigma. Freund's adjuvuant (complete) was obtained from DIFCO Laboratories, Detroit, Michigan. *E. gracilis* strain Z was obtained from Carolina Biological Supply Company, Burlington, North Carolina. All plant material was obtained from fresh market sources. The millipore ultrafiltration cell and Pellicon PSAC membranes were purchased from Millipore. Spectrophotometric measurements were performed on the Perkin-Elmer Coleman 124 spectrophotometer with recorder. Cell counts were determined with a Levy-Hausser counting chamber hemacytometer (Neubauer, improved). Sonication was done using the Biosonik III (Bronwill Scientific, Rochester, New York). Protein was determined by the Biuret [4] and Lowry [5] methods. Chlorophyll content was determined by the methods of Arnon [6] and Vernon [7].

Ferredoxin was prepared to homogeneity from spinach leaves according to a slightly modified procedure of Tagawa and Arnon [8]. Protein concentration was measured by using the molar extinction coefficient of 9.680 M^{-1} cm^{-1} at 420 nm [9]. Ferredoxin NADP-reductase was purified to homogeneity essentially by the method of Forti [10]. The concentration was estimated using the molar extinction coefficient of 10.740 M^{-1} cm^{-1} at 456 nm [10]. The molar extinction coefficient of cytochrome *c* at 550 nm is 19 100 M^{-1} cm^{-1}, from which its concentration was determined. Rabbits (New Zealand white) were immunized subcutaneously with purified ferredoxin (1.0 mg per rabbit) emulsified in complete Freund's adjuvant. Immunization was continued in 10-day intervals and the sera collected 7 days after the second immunization, through the ear. Pre-immune sera yielded no visable precipitate with purified antigen nor inhibition of NADPH-linked reduction of cytochrome *c*.

Stock cultures of *E. gracilis*, strain Z were maintained on agar slants composed of a 2% agar base in an inorganic salt medium according to the procedure of Cramer and Meyers [11]. Vitamin B-12 (5 μg/l) and thiamin-HCl (1 μg/l) were added as supplements. Liquid cultures for study were grown on this medium and aerated with 5% CO_2/95% air under constant illumination according to Matson et al. [12].

Preparation of cell-free extracts

Euglena cells in the late logarithmic phase of growth were collected under sterile conditions by centrifugation. Pelleted cells were washed twice in saline solution (0.9% NaCl), centrifuged and resuspended in a small volume of saline. An aliquot was removed for cell number determination. 2 ml of cell paste was then mixed with 30 ml of acetone (−20 °C) and immediately centrifuged at 39 100×*g* for 10 min. The cells were then subjected to a second acetone treatment, centrifuged and the resulting acetone powder dried in a vacuum desiccator at 5 °C. Cells were normally dried from 1 to 2 days and then stored at −20 °C under vacuum desiccation. Purified spinach ferredoxin incubated in 95% aqueous acetone (22 °C) for 10 min was less than 20% denatured as measured by its cytochrome *c* reductase activity and degree of inhibition by antisera.

Acetone-powdered cells were weighed and the extraction of ferredoxin performed in 0.01 M Tris·HCl, pH 7.5, using a tissue homogenizer. Cell breakage was checked periodically by light microscopy, which indicated virtually complete cell breakage after the final homogenization.

Upon homogenization, the extract was centrifuged at 39 100×*g* for 10 min and the supernatant removed. The pelleted debris was resuspended in a small volume of buffer and homogenized an additional two times. Finally, all supernatant fractions were combined and the homogenizer rinsed in fresh buffer. The combined fractions were then clarified by centrifugation at 39 100×*g* for 30 min. The resulting supernatant was adjusted to a constant volume and assayed directly or concentrated by millipore ultrafiltration using PSAC 1000 molecular weight cut-off membranes. The pelleted debris contained negligible cytochrome *c* reductase activity indicating almost complete extraction of ferredoxin prior to assay or concentration. A 2-min sonication of the debris yielded no additional cytochrome *c* reductase activity.

Deveined leaves were washed in distilled water several times and then blended

in 0.01 M Tris·HCl, 0.25 M sucrose, pH 7.5, for 1 min using a Waring Blender. The resulting homogenate was then poured into a large volume of acetone (−20 °C) and immediately centrifuged at 13 700 × *g* for 30 min. The acetone-powdered tissue was dried under vacuum desiccation. Extraction and concentration was done in the manner previously described for *Euglena*. Cell breakage and solubilization of ferredoxin were checked and found to be complete, in the manner described above.

Measurement of ferredoxin in crude extracts

In order to determine whether or not ferredoxin could be accurately measured in crude extracts an experiment was run using various spinach samples at approximately the same ferredoxin content as determined by the absorbance at 420 nm, but of different purity (40–90 %) measured by $A_{420\,nm}/A_{276\,nm} = 0.49$ as 100 % pure spinach ferredoxin. The results indicate that a ferredoxin content in the linear portion of this curve (0.3–1.5 nmol) can be accurately measured in such samples under the experimental conditions employed. When plant or *Euglena* extracts were measured for ferredoxin, aliquots were used that corresponded to cytochrome *c* reductase activity in this range.

Highly purified ferredoxin-NADP reductase should be used because the reductase possesses a slight cytochrome *c* reductase activity [14]. This activity has been found to increase with samples partially denatured. The plant extracts all possessed a small, nonspecific cytochrome reductase activity that did not require ferredoxin-NADP reductase nor NADPH.

Antibody titration for purified spinach ferredoxin and plant extracts

The activity and titer of the purified antisera was determined from the inhibition of cytochrome *c* reduction, according to the following method. Varying amounts of antisera (0.2–20 mg) were mixed with a constant amount of ferredoxin (0.1–2.5 nmol) and incubated for 1 h at room temperature. Upon completion of precipitation of the antigen · antibody complex, the solutions were adjusted to a volume of 1.4 ml with cold 0.01 M Tris · HCl, pH 7.5, and centrifuged at approx. 2000 rev./min for 10 min in a clinical centrifuge. A 0.7-ml aliquot of the clarified supernatant was removed and placed in a 3 ml cuvette to which was added in final concentration: ferredoxin-NADP reductase, $3.32 \cdot 10^{-9}$ M; cytochrome *c*, $1.45 \cdot 10^{-5}$ M and NADPH, $2.14 \cdot 10^{-5}$ M. The cuvette was adjusted to a final volume of 2.8 ml with 0.01 M Tris · HCl, pH 7.5. The change in absorbance at 550 nm upon the addition of NADPH was recorded by utilizing a 5 mV recorder expansion scale. The initial velocity was determined and the results expressed in terms of nmol cytochrome *c* reduced per min and per cent cytochrome *c* reduction. 100 % reduction was defined as the maximal initial velocity obtained with no antisera added. Titer for the antisera was defined near the equivalence point of the titration curve by extrapolation of the linear portion of the curve (Fig. 1). The best line was established by the method of least squares.

RESULTS

Using the method described above, Fig. 1 indicates that pure spinach ferredoxin, spinach extract and *Euglena* extract preparations behave similarly to anti-

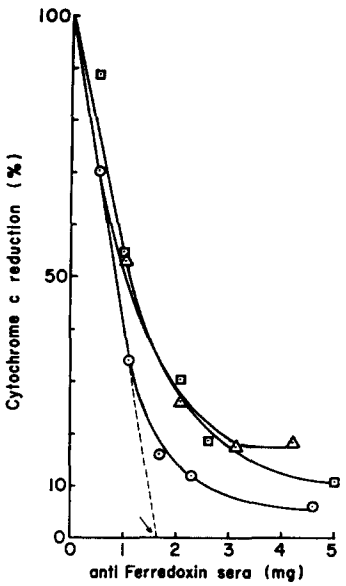

Fig. 1. Titration of ferredoxin with antiferredoxin sera. ○, pure spinach ferredoxin (17.4 μg); △, *Euglena* cell-free extract (300 μg protein); □, spinach extract (400 μg protein). The reaction mixture contained: sample above plus in final concentration $3.32 \cdot 10^{-9}$ ferredoxin-NADP reductase, $1.45 \cdot 10^{-5}$ M cytochrome *c* and $2.14 \cdot 10^{-5}$ M NADPH in 0.01 M Tris · HCl, pH 7.5. Samples were first mixed with antisera and incubated at 22 °C as previously described. The arrow indicates the titer point for purified spinach ferredoxin.

sera against spinach ferredoxin. This agrees with the report that purified *Euglena* ferredoxin can substitute for spinach ferredoxin in the cytochrome *c* reduction [13]. Using the titer points from varying amounts of ferredoxin vs anti ferredoxin sera, a standard curve for spinach ferredoxin may be obtained. Titration of the extracts to this titer point thus allows the concentration of ferredoxin to be determined directly from the graph. Fig. 2 shows titration curves for spinach ferredoxin and lettuce, parsley and broccoli extracts. Table I summarizes the results that were obtained from *Euglena* and the various plant extracts. Each sample represents a separate acetone powder preparation.

DISCUSSION

The classical Hill reactions of NADP and cytochrome *c* photoreductions are dependent upon the components of the photosynthetic system to measure ferredoxin activity. Considerable loss of ferredoxin and other components such as ferredoxin NADP reductase and cytochrome 552 may occur during the isolation of chloroplasts used in these studies (15–17). Addition of ferredoxin to such preparations only partially restores the activity [18]. Recently, it has been demonstrated that other photosynthetic electron transporting molecules may compete with ferredoxin for the reduction of cytochrome *c*. These ferredoxin-independent cytochrome *c* reductions are summarized by San Pietro [19]. As a result of these considerations,

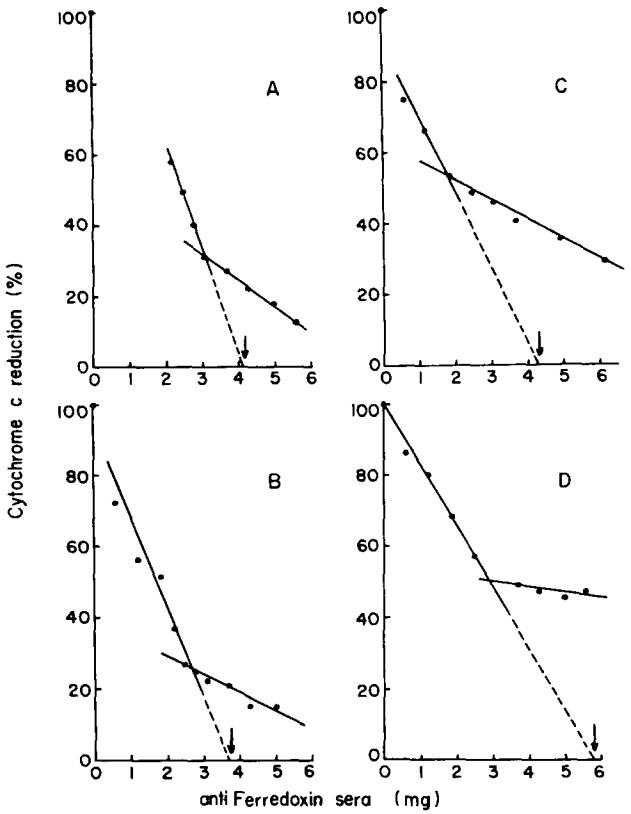

Fig. 2. Titration of ferredoxin with antiferredoxin sera. (A) pure spinach ferredoxin (11.7 μg); (B) parsley extract (111.6 μg protein); (C) lettuce extract (74.3 μg protein); (D) broccoli extract (132.6 μg protein). The reaction conditions are the same as those described in Fig. 1, except different antisera was used. Arrows indicate titer points.

it is difficult to assess the ability of spectrophotometric [1] and oxygen electrode [2, 20] methods that rely upon Hill reactions to measure accurately ferredoxin content.

Haslett et al. [2], in addition to oxygen electrode analysis, used EPR spectroscopy to measure ferredoxin. Such measurements require partial purification of the samples, thus introducing an inherent loss of ferredoxin content. In addition, Arnon reported [21] the isolation and characterization of a new iron-sulfur protein in spinach with similar line shape and g-value to that of ferredoxin. The specificity of this method, therefore, limits the measurement of soluble ferredoxin.

Enzymatic staining techniques on polyacrylamide gels such as the one developed for nitrogenase by Brill et al. [3] is applicable to other iron-sulfur proteins. Quantitation, however, is hindered by color diffusion and gel background that must be resolved during a densiometric trace.

We have utilized the specificity of the antigen · antibody complex together with the sensitivity of a spectrophotometric method to produce a reliable, accurate measure of soluble ferredoxin content in cell free extracts. Although the antibody

TABLE I

THE FERREDOXIN CONTENT OF *EUGLENA* AND VARIOUS PLANT EXTRACTS

The results were calculated from experiments similar to those in Fig. 2. Antiferredoxin sera were standardized as described in the Methods section. Values for ferredoxin are defined in terms of the spinach ferredoxin equivalent. Parenthesis indicates average values for ferredoxin content. Non-heme iron (NHI) was determined according to the method of Massey [25].

Source	Ferredoxin (fd)		Chlorophyll (chl) (μg/mg acetone powder)	Non-heme iron (NHI) (nmol/mg acetone powder)	fd/chl (nmol/mg)	fd/NHI (nmol/nmol)
	(nmol/mg ext. protein)	(nmol/mg acetone powder)				
Spinach (*Spinacia oleracea*)	1.1– 2.6 (1.8)	0.13–0.16 (0.14)	4.32	0.33	34	0.42
Euglena (*E. gracilis strain Z*)	2.1– 5.4 (3.9)	0.33–0.43 (0.38)	36.9	1.08	13	0.40
Parsley (*Petroselinum crispum*)	5.7– 6.1 (5.9)	0.29	2.93		99	
Lettuce* (*Lactuca sativa*)	12.9–15.8 (14.8)	0.80–0.87 (0.84)	1.34		627	
Broccoli* (*Brassica oleracea*)	10.3–11.5 (10.9)	0.62	3.49		178	

* See discussion section for an explanation of these values.

was prepared to spinach ferredoxin, its cross-reactivity with other chloroplast ferredoxins enables the utilization of this method in a variety of biological systems. This aspect is further supported by the finding of Tel-or and Avron [22]. We found that *Euglena*, spinach and parsley cytochrome *c* reductase activities were inhibited to the same degree and in the same manner as a similar amount of purified spinach ferredoxin was with spinach ferredoxin antibody. As a result, we suggest that these ferredoxins have very similar antigenic properties and an accurate measure of their ferredoxin content has been determined. This would agree with the fact that these particular ferredoxins are of similar molecular weight, labile iron-sulfur and amino acid composition and are able to substitute for spinach ferredoxin in cytochrome *c* reduction [23, 24].

In addition, we measured the total non-heme iron content of spinach and *Euglena* extracts (Table I). There appears to be a reasonable approximation to that expected from the ferredoxin content assayed.

Lettuce and broccoli cytochrome *c* reductase activities failed to be inhibited to the same extent. As previously mentioned, such extracts possess not only ferredoxin-dependent cytochrome *c* reductase activity but also a ferredoxin-independent activity. The degree of inhibition by antiferredoxin may not be reflected in

such titrations (Fig. 2 C, D) thus leading to higher estimations of ferredoxin content. Another alternative would be the lack of specificity of spinach ferredoxin antibody towards these plant ferredoxins. We conclude that with such uncertainty in these measurements the spinach ferredoxin antibody titration may not be accurate for lettuce and broccoli.

Ferredoxin content (nmol/mg chlorophyll) has been determined for each plant (Table I). Arnon [8] first estimated the molar ratio chlorophyll: ferredoxin at 400 : 1 (approx. 2.8 nmol ferredoxin/mg chlorophyll) for spinach. Whately [2] obtained a similar value for bean leaves. Our method yields a value of 33 nmol ferredoxin/mg chlorophyll which agrees with that of Park and Biggins [26] who found 24 nmol ferredoxin/mg chlorophyll. The lower values reported by Arnon and Whately may be due to loss of ferredoxin upon its purification, from which these values were obtained.

Perini et al. [27] determined that *Euglena* ferredoxin content was similar to that reported by Arnon for spinach. They assumed, however, 10 gatoms of iron per mol of ferredoxin. Since it has been established that *Euglena* ferredoxin contains 2 gatoms of iron per mol [24] their value rises to 14 nmol ferredoxin/mg chlorophyll. This is very close to the value obtained by immunoassay of 13 nmol per mg chlorophyll.

ACKNOWLEDGEMENTS

This study was supported by a Research Grant from the National Institutes of Health (AM-12713). A part of this work was done for the partial fulfillment of the requirements of the Doctor of Philosophy (R.S.M.). R.S.M. is a recipient of the Knoller Fellowship in Chemistry, Wayne State University.

REFERENCES

1 Trebst, A. (1972) Methods in Enzymology (San Pietro, A., ed.), Vol. XXIV, Part B, pp. 146–165, Academic Press, New York
2 Haslett, B. G., Cammack, R. and Whatley, F. R. (1973) Biochem. J. 136, 697–703
3 Brill, W. J., Westphal, J., Stieghortst, M., Davis, L. C. and Shah, V. K. (1974) Anal. Biochem. 60, 237–241
4 Gornall, A. G., Baradwill, C. S. and David, M. M. (1949) J. Biol. Chem. 177, 751–766
5 Lowry, O. H., Rosenberg, N. J., Farr, A. L. and Randall, R. S. (1951) J. Biol. Chem. 193, 265–275
6 Arnon, D. I. (1949) Plant Physiol. 24, 1–15
7 Vernon, L. P. (1960) Anal. Chem. 32, 1144–1150
8 Tagawa, K. and Arnon, D. I. (1962) Nature 195, 537–543
9 Tagawa, K. and Arnon, D. I. (1968) Biochim. Biophys. Acta 153, 602–613
10 Forti, G. (1971) Methods in Enzymology (San Pietro, A., ed.), Vol. XXII, Part A, pp. 447–451, Academic Press, New York
11 Cramer, M. and Meyers, J. (1952) Arch. Microbiol. 17, 384–402
12 Matson, R. S., Fei, M. and Chang, S. B. (1970) Plant Physiol. 45, 531–532
13 Smillie, F. M. (1968) Enzymology of Euglena (Buetow, D. E., ed.), Vol. II, pp. 16–17, Academic Press, New York
14 Nakamura, S. and Kimura, T. (1972) J. Biol. Chem. 247 (20), 6462–6468
15 Wildner, G. F. and Hauska, G. (1974) Arch. Biochem. Biophys. 164, 127–135
16 Graham, D. and Smillie, R. M. (1971) Methods in Enzymology (San Pietro, A., ed.), Vol. XXIII, Part A, pp. 228–242, Academic Press, New York

17 Boger, P. (1971) Methods in Enzymology (San Pietro, A., ed.), Vol. XXIII, Part A, pp. 242–248, Academic Press, New York
18 Biggins, J. (1967) Plant Physiol. 42, 1442–1456
19 Siedow, J. N. and San Pietro, A. (1974) Arch. Biochem. Biophys. 164, 145–155
20 Allen, J. P. and Hall, D. O. (1974) Biochem. Biophys. Res. Commun. 58 (3), 579–585
21 Malkin, R., Aparicio, P. J. and Arnon, D. I. (1974) Proc. Natl. Acad. Sci. U.S. 71 (6), 2362–2366
22 Tel-or, E. and Avron, M. (1974) Eur. J. Biochem. 47, 417–421
23 Wickramasingne, R. H. and McIntosh, E. N. (1974) Enzyme 17, 210–266
24 Mitsui, A. (1971) Biochim. Biophys. Acta 243, 447–456
25 Miller, R. W. and Massey, V. (1965) J. Biol. Chem. 240, 1453–1465
26 Park, R. and Biggins, J. (1964) Sci. 144, 1009–1011
27 Perini, F., Kamen, M. D. and Schiff, J. A. (1964) Biochim. Biophys. Acta 88, 74–90