Physicochemical Properties of Ferredoxin from *Chlamydomonas reinhardii*

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Ferredoxin from *Chlamydomonas reinhardii* has been purified to electrophoretic homogeneity by an easy and fast procedure with a high yield (25–30 mg/250 g wet weight of cells). An average molecular weight of 11800 was calculated from sedimentation coefficient (1.70 S) and Stokes radius (1.75 nm) data, sodium dodecyl sulfate-electrophoresis, and amino acid composition. Absorption spectrum showed maxima at 276, 330, 420 and 460 nm in the oxidized form, with an absorption ratio (A_{420}/A_{276}) of 0.54 and an extinction coefficient of 8.38 $mM^{-1} \cdot cm^{-1}$ at 420 nm. Reduced ferredoxin showed a single peak at 276 nm with shoulders at 284, 310, 390, 469 and 537 nm and at liquid helium temperatures gave EPR signals at g = 1.877, 1.951 and 2.045. The protein has an isoelectric point of 3.30, and one (2Fe–2S)-cluster per molecule with a midpoint potential, at pH 7.5, of −410 mV (n=1). The molecule of *C. reinhardii* ferredoxin consists of 95–99 amino acid residues which includes the full complement of amino acids, being alanine the most abundant.

Introduction

In higher plants, green algae and blue-green algae, ferredoxin is a small molecule (molecular weight about 12000) containing a two iron-two labile sulfur-cluster, which participates in chloroplasts photosynthetic electron transfer due to its high negative redox potential [1–4]. The relative ease of purification of ferredoxins has allowed to know the physicochemical properties, amino acid composition and amino acid sequence of many plant and algal ferredoxins [3, 5–12].

Chlamydomonas reinhardii is an haploid green alga of relatively well-known genetics [13], whose ferredoxin has been used in genetic transcription and translation studies as marker of chloroplast protein [14], in immunological approaches to its biosynthetic process [15], and in characterizations of ferredoxins by mapping of tryptic peptides [16]. Although this protein has been previously purified [8, 15, 17], its physicochemical properties remain to be described.

In this work we characterize physicochemically the *C. reinhardii* ferredoxin. The protein of M_r = 11800 contains one (2Fe–2S)-cluster per molecule, with a redox midpoint potential of −410 mV, and the full complement of amino acids. By the first time, new absorption shoulders are reported for a plant-type ferredoxin in its reduced form.

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Materials and Methods

Growth conditions and preparation of extracts

C. reinhardii cells, strain 21 *gr* (from Dr. Ruth Sager, Sidney Farber Cancer Ctr.), were grown at 25 °C in liquid medium containing 10 mM NH_4Cl [18], bubbled with air supplemented with 5% (v/v) CO_2 under continuous light (15–20 $W.m^{-2}$). Cell-free extracts were prepared by freezing and thawing the cells in 50 mM potassium phosphate buffer, pH 7.5 (standard buffer) as described [19].

Purification procedure

Purification of ferredoxin was carried out at 0–4 °C by the following procedure: 1. 2% (w/v) protamine sulfate (pH 7.5) was added dropwise to the crude extract (1 ml/10 ml extract) with gentle stirring. After 15 min, the suspension was centrifuged at 16000 × g, 15 min. 2. The resulting supernatant was applied to a DEAE-Sephacel column (3 × 40 cm) equilibrated with standard buffer, and ferredoxin eluted with the same buffer but 0.5 M in NaCl. 3. Fractions containing ferredoxin were pooled and di-

luted 5-fold with standard buffer and applied to a second DEAE-Sephacel column (2 × 20 cm) equilibrated with the same buffer. Ferredoxin was eluted with 300 ml of a linear gradient of NaCl (0.2–0.7 M) in standard buffer. Fractions containing ferredoxin were pooled, dialyzed against standard buffer during 12 h, and concentrated by using a DEAE-Sephacel column (1 × 8 cm) as in step 2. The eluate was dialyzed against standard buffer during 12 h and used as source of the protein.

Analytical determinations

Labile sulfide was determined by the method of Siegel *et al.* [20] using an absorbance coefficient of 31.2 $mM^{-1} \cdot cm^{-1}$ at 670 nm. Iron was analysed according to Massey [21]. An extinction coefficient of 9.34 $mM^{-1} \cdot cm^{-1}$ was found for the iron-*o*-phenan – throline complex at 510 nm, using cytochrome *c* as standard. Protein content was estimated from the average of values obtained by the methods of Lowry *et al.* [22], Bradford [23] and biuret [24], with bovine serum albumin as standard. Cytochrome *c*, carbonic anhydrase, trypsin inhibitor, ovalbumin and bovine serum albumin were determined by measuring the absorbance at 280 nm.

Absorption and EPR spectra

Absorption spectra were recorded at 25 °C in special anaerobic quartz cuvettes of 1-cm path-light with a DW-2a™ UV-VIS AMINCO spectrophotometer. EPR spectra were recorded in a BRUKER-200 tt spectrometer at 10 K (Prof. A.V. Xavier, I.S.T., Univ. Nova, Lisboa).

Electrophoresis

Analytical electrophoresis of purified ferredoxin was performed at 4 °C in 7.5% polyacrylamide gels according to Jovin *et al.* [25]. Electrophoresis in sodium dodecyl sulfate was performed at 25 °C in 10% polyacrylamide gels according to Weber and Osborn [26]. Standards of known molecular weight were: cytochrome *c*, 12400; lysozyme, 14400; soybean trypsin inhibitor, 21500; carbonic anhydrase, 31000; ovalbumin, 45000; bovine serum albumin, 66000 and phosphorylase *b*, 92500. Proteins were located by staining with 1% (w/v) coomassie brilliant blue R-250 in 7% (v/v) acetic acid.

Determination of the isoelectric point

Isoelectric focusing was carried out at 4 °C in 4.5% polyacrylamide gels containing 1% (w/v) Ampholine, pH 3.5–10, according to Maurer [27]. The ferredoxin band (50 μg) was directly located in the gels by its brown colour. pH of each 0.5 cm-portions of the gel, ground in 1-ml distilled water and incubated overnight, was measured with an ORION 701A pH-meter.

Determination of Stokes radius and sedimentation coefficient

Stokes radii were determined according to Siegel and Monty [28] by using a Sephadex G-75 column (1.5 × 20.8 cm) equilibrated with standard buffer. 0.5-ml samples containing ferredoxin (0.7 mg) and/or standard proteins (cytochrome *c*, 1.74 nm; trypsin inhibitor, 2.25 nm and ovalbumin, 2.76 nm) were used. Calculations were carried out as reported [29]. Sedimentation coefficients were determined by sucrose density gradient centrifugation as described by Martin and Ames [30]. Linear gradients of 5–12% (w/v) sucrose in standard buffer were used. 0.1-ml samples containing ferredoxin (1.5 mg) and/or the marker proteins (cytochrome *c*, 1.83 S; carbonic anhydrase, 2.85 S and ovalbumin, 3.55 S) were centrifuged at 43000 r.p.m., 19 h, in a SW-50.1 Ti rotor. After centrifugation, 3-drops fractions were collected from the bottom of the tubes.

Titration of sulfhydryl groups and amino acid analysis

Total sulfhydryl equivalents of ferredoxin were titrated with *p*-hydroxymercuribenzoate according to Boyer [31]. For amino acid analysis, samples were hydrolysed at 115 °C for 24, 48 and 72 h in 4 M methanesulfonic acid containing 0.2% (w/v) 3-(2-aminoethyl)indol in evacuated sealed ampoules. The analyses were carried out with a CHROMASPECK-J-180 autoanalyzer (Dr. J. L. Periago, Univ. Granada) using *nor*-Leu as internal standard and *o*-ftaldehyde as fluorogenic reagent. Thr and Ser content was calculated from extrapolation to zero time of the obtained values. Val, Met, Ile and Phe content was obtained from averaged values at 48 and 72-h hydrolysis. Trp content of separate samples was determined spectrophotometrically by the method of Edelhoch [32]. Cys content was deduced from sulfhydryl groups titration data. For other amino acid residues,

averaged values at the different times of hydrolysis were considered. The number of amino acid residues per mol of ferredoxin and the minimum molecular weight were calculated by the procedure of Thornber and Olson [33].

Potentiometric titration

Redox titration was performed in anaerobic quartz cuvettes, at 25 °C, using a DW-2a™ UV-VIS AMINCO spectrophotometer and a BECKMAN 4 500 potentiometer with a combined Pt–Ag/AgCl Ingold electrode (E_o = 222 mV at 25 °C). The reaction mixture was degassed and bubbled with N_2 previously passed through pyrogallol. Absorbance changes at 420 nm and redox potential were measured simultaneously after adding aliquots of a 20 mM dithionite solution, anaerobically prepared in 0.5 M bicarbonate buffer (pH 8.3). Redox mediators used were: 2 μM benzyl viologen (E'_o, pH 7.0 = −360 mV) and 2 μM methyl viologen (E'_o, pH 7.0 = −440 mV).

Results and Discussion

A typical purification of *C. reinhardii* ferredoxin is shown in Table I. The purified preparations were homogeneous since electrophoresis of high quantities of protein (up to 120 μg) always rendered a unique band of protein which corresponded to the brown band of ferredoxin (results not shown). We have detected a single type of ferredoxin in *C. reinhardii* along the purification procedures and analytical techniques. However, some higher plants [34–36] and blue-green algae [10, 37] contain two molecular species of ferredoxin. After the second DEAE-Sephacel chromatography, ferredoxin was eluted with an absorption ratio (A_{420}/A_{276}) of 0.65 (Result not shown). Dialysis of samples lowered this ratio to 0.54 (Table I), probably because of denaturation of the chromophore group. A yield of 25.2 mg of pure protein was obtained from 225 g of cells (wet weight), which is significantly higher than those reported for different green algae and higher plants [6, 9, 38, 39] but not so high as that for *Dunaliella salina* [40]. This fact together with the simple freezing-thawing treatment used to disrupt the cells [19] render *C. reinhardii* a good organism of choice to obtain quickly high amounts of algal ferredoxin.

Purified ferredoxin showed a typical spectrum of oxidized ferredoxins of the plant type [3, 15, 17], with absorption maxima at 276, 330, 420 and 460 nm and a shoulder at 284 nm (Fig. 1, A). Under strict anaerobic conditions, small amounts of sodium dithionite reduced quickly ferredoxin causing the disappearance of the peaks at 330, 420 and 460 nm to-

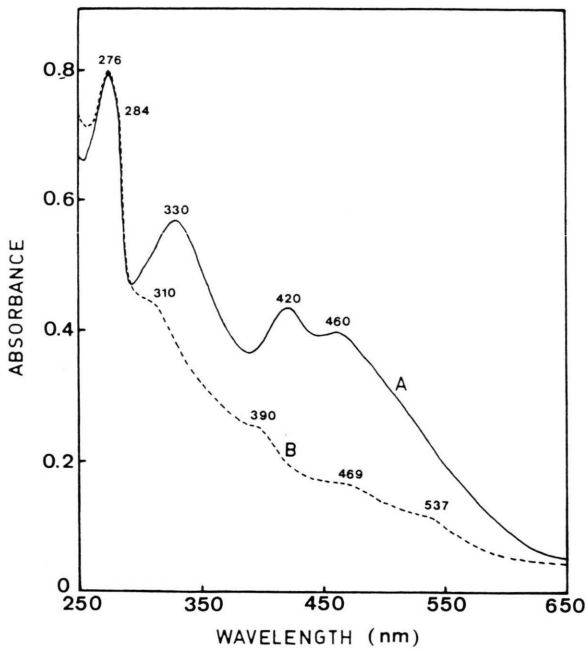

Fig. 1. Absorption spectra of *C. reinhardii* ferredoxin. A: absorption spectrum, at +270 mV, of purified ferredoxin (0.66 mg/ml) in 50 mM phosphate buffer, pH 7.5. B: absorption spectrum, at −470 mV, of ferredoxin reduced with 0.1 μmol dithionite anaerobically prepared in 0.5 M bicarbonate buffer, pH 8.3. Previously, the native protein was degassed and bubbled with N_2 as detailed in Materials and Methods.

Step	Volume [ml]	Protein [mg]	A_{420}	A_{276}	A_{260}	A_{420}/A_{276}
1. Crude extract	820	3331	0.71	9.73	14.91	0.07
2. Protamine sulfate	820	2404	0.52	4.15	5.53	0.13
3. 1st DEAE-Sephacel	90	41	0.20	0.50	0.60	0.40
4. 2nd DEAE-Sephacel	30	25	0.44	0.81	0.70	0.54

Table I. Purification of ferredoxin from *Chlamydomonas reinhardii*.

Details of the purification method are described in Materials and Methods.

Table II. Physicochemical properties of ferredoxin from *Chlamydomonas reinhardii*.

Stokes radius (α)	1.75 nm
Sedimentation coefficient ($S_{20,w}$)	1.70 S
Molecular weight:	
−from α and $S_{20,w}$	12200
−from electrophoresis in sodium dodecyl sulfate	12600
−from amino acid composition	10600
Average value	11800
Frictional ratio	1.16
Extinction coefficient at 420 nm	8.38 $mM^{-1} \cdot cm^{-1}$
A_{420}/A_{276}	0.54
Isoelectric point (pI)	3.30
Midpoint potential (E'_o, pH 7.5)	−410 mV
Number of electrons involved (n):	
−analytical data	1.36
−deduced from data	1
Labile sulfide (mol/mol protein):	
−analytical data	1.55
−deduced from data	2
Iron (mol/mol protein):	
−analytical data	1.99
−deduced from data	2

gether with the appearance of new shoulders at 310, 390, 469 and 537 nm (Fig. 1, B), some of which, up to now, have not been reported for a chloroplast ferredoxin. Reduced spinach ferredoxin shows a marked peak at 276 nm, a shoulder at 312 nm and a broad shoulder between 450 and 470 nm [41], but spectra of reduced ferredoxin from other sources have not been analysed in the ultraviolet region [2–4, 6, 8, 9]. Reduced ferredoxin is auto-oxidizable since its native absorption spectrum was restored after oxidation with air (Result not shown). Assuming a molecular weight of 11800, an extinction coefficient of 8.38 $mM^{-1} \cdot cm^{-1}$ was calculated at 420 nm (Table II).

Oxidized ferredoxin did not show EPR signals. After reduction with sodium dithionite, the protein showed an EPR spectrum with rhombic symmetry and signals at g = 1.877, 1.951 and 2.045 (Fig. 2), typical of (2Fe–2S)-clusters common to all known ferredoxins of the plant type [1–4, 42].

Samples of ferredoxin whose protein content was estimated by three different methods had an average content of 1.99 atoms of iron and 1.55 atoms of acid-labile sulphur per molecule (Table II), thus corroborating the presence of a (2Fe–2S)-cluster in *C. reinhardii* ferredoxin, shown by EPR spectra. The protein was also titrated with *p*-hydroxymercuribenzoate, rendering 8 sulfhydryl equivalents per mol (Result not shown). Since 1 mol of ferredoxin contains 2 mol of acid-labile sulfide (Table II) which react with 4 equivalents of *p*-hydroximercuribenzoate [31], the remaining 4 sulfhydryl equivalents correspond to the 4 mol of cysteine in the protein molecule.

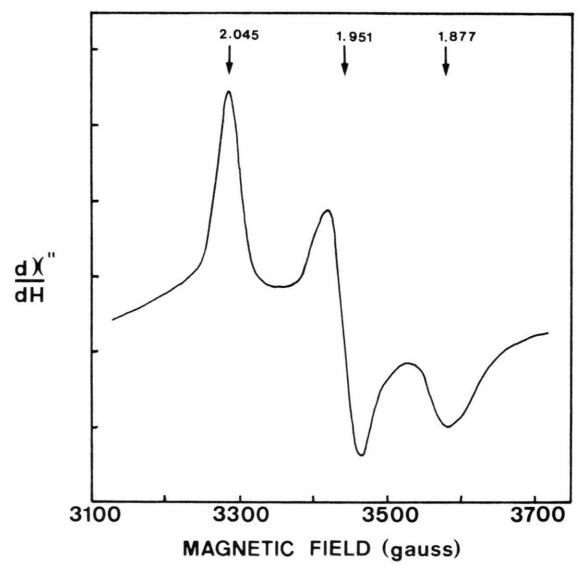

Fig. 2. EPR spectrum of *C. reinhardii* ferredoxin. The spectrum of purified protein (1 mg/ml) in 50 mM phosphate buffer, pH 7.5, reduced with crystals of dithionite, was recorded at 10 K (microwave frequency = 9.404 GHz). Arrows indicate g values.

The amino acid composition of *C. reinhardii* ferredoxin is shown in Table III and compared with that of ferredoxins from two other green algae, one blue-green alga and spinach. Although the resemblances

Table III. Amino acid composition of *Chlamydomonas reinhardii* ferredoxin and comparison with several 2 Fe-ferredoxins.

Amino acid	Number of residues per molecule						
	Chlamydomonas reinhardii[a]		*Scenedesmus* sp.[b]	*Euglena gracilis*[c]	*Synechococcus* sp.[d]	Spinach[e]	
	f	g				I	II
Lys	4.5	4–5	4	5	4	4	4
His	0.6	1	1	1	0	1	1
Arg	1.6	1–2	1	1	3	1	1
Asx	10.2	10	12	14	12	13	8
Thr	8.7	9	10	9	7	8	8
Ser	8.5	8–9	8	8	6	7	8
Glx	10.9	11	10	9	16	13	15
Pro	2.1	2	4	4	4	4	3
Gly	9.3	9	7	7	6	6	9
Ala	12.7	13	10	8	6	9	9
Cys*	4	4	6	6	5	5	5
Val	4.8	5	5	6	7	7	7
Met	0.9	1	1	0	0	0	2
Ile	2.6	2–3	3	4	4	4	4
Leu	6.9	7	7	7	10	8	6
Tyr	5	5	4	1	4	4	4
Phe	2.3	2	3	3	3	2	1
Trp*	1	1	0	0	0	1	1
Total	95–99	95–99	96	93	97	97	97

* Details are given in Materials and Methods. [a]Present work. [b]Ref. 6. [c]Ref. 8. [d]Ref. 12. [e]Refs. 34, 43. [f]Found value. [g]Integral value.

among them are significant, it is noteworthy that *C. reinhardii* ferredoxin contains 95–99 amino acid residues per molecule and shows the full complement of amino acids with a high content of alanine.

Stokes radius of 1.75 nm and sedimentation coefficient of 1.70 S were found for purified ferredoxin (Table II). From these data and assuming an apparent specific volume of 0.725 $cm^3 \cdot g^{-1}$, a molecular weight of 12200 has been calculated according to the method of Siegel and Monty [28]. In sodium dodecyl sulfate-polyacrylamide gel electrophoresis, ferredoxin was shown as a single band of molecular weight 12600, very close to that found above, which indicates that the protein is a monomer (Result not shown). Low molecular weight proteins use to deviate about 10% in mobility rate [44], which explains the higher value of molecular weight found for ferredoxin by this technique. A minimum molecular weight of 10600 was calculated from the amino acid composition and iron and labile sulfide content (Table II), according to Thornber and Olson [33]. An average molecular weight of 11800 has been used in all calculations. Accordingly, a frictional ratio of 1.16 was calculated, which suggests an almost-spherical shape for the molecule (Table II).

The isoelectric point (3.30) of *C. reinhardii* ferredoxin (Table II), its midpoint redox potential, at

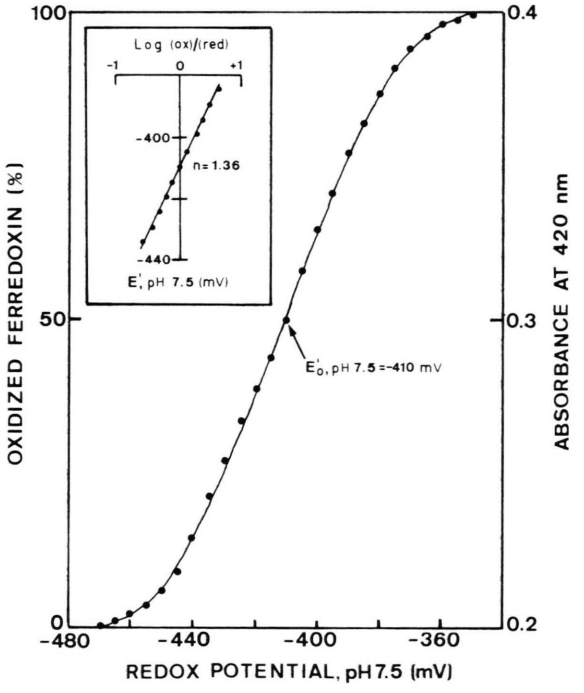

Fig. 3. Reductive titration of *C. reinhardii* ferredoxin and its corresponding Nernst's plot (inset). 3-ml samples of 56 μM ferredoxin in 50 mM phosphate buffer, pH 7.5, containing redox mediators were titrated as described in Material and Methods.

pH 7.5, (−410 mV) and its behaviour as one-electron carrier (Fig. 3) are in agreement with data reported for other (2Fe−2S)-cluster-containing ferredoxins [2, 3, 5, 45, 46].

The results presented in this work indicate that *C. reinhardii* ferredoxin shows most of the characteristics of plant-type ferredoxins. The purified protein, when reduced with dithionite, maintained its physiological properties acting as an effective electron donor of ferredoxin-glutamate synthase (EC 1.4.7.1) of *C. reinhardii* [47]. The relative ease to obtain high amounts of this protein enables further studies on its complete structure.

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[1] R.H. Wickramasinghe and E.N. McIntosh, Enzyme **17,** 210 (1974).
[2] R.H. Wickramasinghe, Enzyme **17,** 227 (1974).
[3] B.B. Buchanan and D.I. Arnon, Adv. Enzymol. **33,** 119 (1970).
[4] D.O. Hall, K.K. Rao, and R. Cammack, Sci. Prog. Oxf. **62,** 285 (1975).
[5] K. Tagawa and D.I. Arnon, Biochim. Biophys. Acta **153,** 602 (1968).
[6] H. Matsubara, J. Biol. Chem. **243,** 370 (1968).
[7] H. Matsubara and R.M. Sasaki, J. Biol. Chem. **243,** 1732 (1968).
[8] A. Mitsui, Biochim. Biophys. Acta **243,** 447 (1971).
[9] J.G. Huisman, M.G.Th. Gebbink, P. Modderman, and D. Stegwee, Planta **137,** 97 (1977).
[10] K.G. Hutson, L.J. Rogers, B.G. Haslett, Boulter, D., and R. Cammack, Biochem. J. **172,** 465 (1978).
[11] M.M. Werber and M. Mevarech, Arch. Biochem. Biophys. **187,** 447 (1978).
[12] T. Hase, H. Matsubara, H. Koike, and S. Katoh, Biochim. Biophys. Acta **744,** 46 (1983).
[13] G.A. Hudock and H. Rosen, The Genetics of Algae (R.A. Lewin, ed.), p. 29, Blackwell Sci. Publications, Oxford 1976.
[14] J.J. Armstrong, S.J. Surzycki, B. Moll, and R.P. Levine, Biochemistry **10,** 692 (1971).
[15] J.G. Huisman, I. Touw, P. Liebregts, and A. Bernards, Planta **145,** 351 (1979).
[16] J.G. Huisman, S. Stapel, and M.G.Th. Gebbink, Anal. Biochem. **90,** 501 (1978).
[17] D.S. Gorman and R.P. Levine, Plant Physiol. **41,** 1643 (1966).
[18] N. Sueoka, K.S. Chiang, and J.R. Kates, J. Mol. Biol. **25,** 47 (1967).
[19] E. Fernández and J. Cárdenas, Mol. Gen. Genet. **186,** 164 (1982).
[20] L.M. Siegel, M.J. Murphy, and H. Kamin, J. Biol. Chem. **10,** 251 (1973).
[21] V. Massey, J. Biol. Chem. **229,** 763 (1957).
[22] O.H. Lowry, N.J. Rosebrough, A.L. Farr, and R.J. Randall, J. Biol. Chem. **193,** 265 (1951).
[23] M.M. Bradford, Anal. Biochem. **72,** 248 (1976).
[24] A.G. Gornall, C.J. Bardawill, and M.M. David, J. Biol. Chem. **177,** 751 (1949).
[25] T. Jovin, A. Chrambach, and M.A. Naughton, Anal. Biochem. **9,** 351 (1964).
[26] K. Weber and M. Osborn, J. Biol. Chem. **244,** 4406 (1969).
[27] H.R. Maurer, Disc Electrophoresis and related techniques of polyacrylamide gel electrophoresis, p. 132, Walter de Gruyter, Berlin 1971.
[28] L.M. Siegel and K.J. Monty, Biochim. Biophys. Acta **112,** 346 (1966).
[29] T.C. Laurent and J. Killander, J. Chromatogr. **14,** 317 (1964).
[30] R.G. Martin and B.N. Ames, J. Biol. Chem. **236,** 1372 (1961).
[31] P.D. Boyer, J. Am. Chem. Soc. **76,** 4331 (1954).
[32] H. Edelhoch, Biochemistry **6,** 1948 (1967).
[33] J.P. Thornber and J.M. Olson, Biochemistry **7,** 2242 (1968).
[34] Y. Takahashi, T. Hase, K. Wada, and H. Matsubara, J. Biochem. **90,** 1825 (1981).
[35] R.M. Nalbandyan, Biokimiya **41,** 188 (1976).
[36] S. Wakabayashi, T. Hase, K. Wada, H. Matsubara, K. Suzuki, and S. Takaichi, J. Biochem. **83,** 1305 (1978).
[37] R. Cammack, K.K. Rao, C.P. Bargeron, K.G. Hutson, P.W. Andrew, and L.J. Rogers, Biochem. J. **168,** 205 (1977).
[38] R. Thauer, H. Schirrmacher, W. Schymanski, and P. Schönheit, Z. Naturforsch. **33,** 495 (1978).
[39] B.B. Buchanan and D.I. Arnon, Methods in Enzymology, **Vol. XXIII** (A. San Pietro, ed.), p. 413, Academic Press, New York 1971.
[40] T. Hase, H. Matsubara, A. Ben-Amotz, K.K. Rao, and D.O. Hall, Phytochemistry **19,** 2065 (1980).
[41] K. Tagawa and D.I. Arnon, Nature **195,** 537 (1962).
[42] D.O. Hall and K.K. Rao, Encyclopaedia of Plant Physiology, **Vol. 5** (A. Trebst and M. Avron, eds.), p. 206, Springer, Berlin 1977.
[43] Y. Takahashi, T. Hase, K. Wada, and H. Matsubara, Plant Cell Physiol. **24,** 189 (1983).
[44] K. Weber, J.R. Pringle, and M. Osborn, Methods in Enzymology, **Vol. XXVI** (C.H.W. Hirs and S.N. Timasheff, eds.), p. 3, Academic Press, New York 1972.
[45] J.E. Dutton and L.J. Rogers, Biochim. Biophys. Acta **537,** 501 (1978).
[46] B. Ke, W.A. Bulen, E.R. Shaw, and R.H. Breeze, Arch. Biochem. Biophys. **162,** 301 (1974).
[47] F. Galván, A.J. Márquez, and J.M. Vega, Planta **168,** 170 (1984).