Plant Physiol. (1982) 70, 1541–1543
0032-0889/82/70/1541/03/$00.50/0

Limiting Factors in Photosynthesis

III. EFFECTS OF IRON NUTRITION ON THE ACTIVITIES OF THREE REGULATORY ENZYMES OF PHOTOSYNTHETIC CARBON METABOLISM

Received for publication April 23, 1982 and in revised form July 8, 1982

SCOTT E. TAYLOR, NORMAN TERRY, AND ROBERT P. HUSTON
Department of Plant and Soil Biology, University of California, Berkeley, California 94720

ABSTRACT

When Fe was withheld from sugar beets (*Beta vulgaris* L. CV F58-44H1), the activities of NADP-glyceraldehyde-3-phosphate dehydrogenase, fructose 1,6-bisphosphatase, and ribulose 5-phosphate kinase were not diminished, while chlorophyll per area was decreased by 75%. On resupplying Fe, chlorophyll per area increased to control levels within 5 days, whereas the activities of the three enzymes remained approximately constant. These results support the view advanced earlier (Terry 1980 Plant Physiol 65: 114–120) that the photosynthetic effects of Fe deprivation are mediated by changes in the lamellar components of chloroplasts and not by changes in stromal enzymes involved in photosynthetic carbon reduction.

In an earlier paper of this series, evidence was presented that Fe stress influenced photosynthesis mainly by altering the capacity of leaves for light harvesting and electron transport (photochemical capacity) (23). Subsequently, Spiller and Terry (19) showed that Fe stress reduced the chloroplast content of Chl *a*, Chl *b*, P_{700}, and Cyt *f* without changing the ratios of Chl/P_{700} or Chl/Cyt *f* and that these events were accompanied by a drastic reduction in the thylakoid system. Based on the fact that severe Fe stress reduced RuBP[1] carboxylase extractable activity by 30% while reducing Chl (*a* + *b*), P_{700}, and Cyt *f* and photosynthesis by more than 90%, it was suggested that Fe stress may have relatively little effect on the stromal enzymes involved in photosynthetic carbon reduction, and if so, that Fe stress could be utilized as an experimental technique to study photosynthetic limitation (23).

The objective of the present investigation was to test the hypothesis that the influence of Fe nutrition on the enzymes of photosynthetic carbon reduction was small relative to the effects on the lamellar components. The approach used was to determine the influence of Fe nutrition on the activity of three important enzymes of the Calvin cycle: GPDHase, FBPase, and RPKase. These enzymes represent possible regulatory steps in CO_2 assimilation: GPDHase catalyzes the reductive step that requires light-generated production of NADPH; FBPase has been suggested to have a pivotal role in the regulation of RuBP carboxylase (6); and RPKase catalyzes the regeneration of the CO_2 acceptor, RuBP, and may be important in the regulation of photosynthetic carbon reduction (8).

[1] Abbreviations: RuBP, ribulose1,5-bisphosphate; FBPase, fructose 1,6-bisphosphatase (EC 3.13.11); GPDHase, NADP-glyceraldehyde-3-phosphate dehydrogenase (EC 1.2.1.13); PRKase, ribulose-5-phosphate kinase (EC 2.7.1.19).

MATERIALS AND METHODS

Plant Culture. Sugar beets (*Beta vulgaris* L. cv F58-44H1) were grown hydroponically in growth chambers as described previously (22). Iron-deficient plants were obtained by transferring to culture solutions without Fe for a period of 7 d. Iron resupply studies were carried out by transferring Fe deficient plants to half Hoagland solution containing ferric-sodium EDTA. All determinations were done with rapidly expanding leaves as described previously (23).

Chloroplast Isolation. Leaves were harvested 2 to 3 h after the start of the photoperiod, and discs removed for determination of leaf Chl content. The remaining leaf material (after removing the mid-rib and petiole) was lowered in temperature by bathing in ice-cold distilled H_2O for 5 min; three to four leaves (approximately 15 g of tissue) were used for each isolation.

Chloroplasts were isolated at 2°C by a 5 s homogenization in a Waring blender using the isolation medium: 20 mM Tricine (pH 7.6), 0.4 M sorbital, 10 mM NaCl, 10 mM $MgCl_2$, 2 mM $CaCl_2$, 2 mM $MnCl_2$, 5 mM EDTA, 5 mM ascorbic acid, and 0.1% w/v BSA (Sigma, type V); the ratio of isolation medium to leaf tissue was 3:1 (v/w). The homogenate was filtered through six layers of nylon mesh and the plastids pelleted at 400*g* for 2 min. The pellet was gently washed with isolation medium, then resuspended in 2.5 ml of hypotonic solution: 10 mM Tricine (pH 8.0), 10 mM $MgCl_2$, 2 mM NaCl, and 2 mM ascorbic acid. Thorough rupture of the chloroplast envelopes was ensured by sonication for 5 s with a Branson Sonifier Cell Disrupter 200. Soluble enzymes were separated from chloroplast membranes by centrifugation at 45,000*g* for 15 min.

To determine and correct for Chl of broken chloroplasts, the percentage of intact chloroplasts present after the initial centrifugation was determined by phase contrast microscopy. Chl was determined as described previously (2, 21).

Enzyme Activity. Chloroplast GPDHase activity was determined as described by Latzko and Gibbs (19) and Wolosiuk and Buchanan (24). The enzyme was activated by the addition of 0.5 ml of a solution containing 0.2 M Tricine (pH 8.4) and 20 mM ATP to 0.1 ml of the solution containing the extracted enzymes. After 5 min of activation at 25°C, we added 0.75 ml of reaction medium (62.5 mM Tricine [pH 8.4], 16 mM $MgCl_2$, 7.8 mM ATP, and 0.2 mM NADPH). The enzymic reaction was initiated by the addition of 0.1 ml of 50 mM glycerate 3-P and the oxidation of NADPH monitored at 340 nm.

FBPase activity was determined as described by Nishizawa *et al.* (11). The enzyme was activated by preincubation of 0.1 ml of enzyme extract with 0.35 ml of reaction medium (0.14 M Tricine [pH 7.9], 14 mM $MgCl_2$, and 7 mM DTT). After activating for 10 min at 25°C, the reaction was initiated by the addition of 0.01 ml of 60 mM fructose-1,6-bisphosphate. The reaction was run at 26°C, and terminated after 15 min by the addition of 0.5 ml of 10% (w/v) TCA. This solution was cleared by centrifugation for 10

Table I. *Rates of Chloroplast NADP-Glyceraldehyde-3-Phosphate Dehydrogenase, Chloroplast Fructose Bisphosphatase, and Ribulose 5-Phosphate Kinase Extracted from Control and Iron-Deficient Leaves*

	Control Plants		Iron-Deficient Plants	
	$\mu mol\ h^{-1}\ mg^{-1}$ Chl	$\mu mol\ h^{-1}\ cm^{-2}$	$\mu mol\ h^{-1}\ mg^{-1}$ Chl	$\mu mol\ h^{-1}\ cm^{-2}$
GPDHase	250 ± 69	15.2 ± 3.6	2270 ± 380	25.0 ± 7.8
FBPase	139 ± 51	10.5 ± 6.6	914 ± 78	14.1 ± 2.2
RPKase[a]	158 ± 24	7.9 ± 1.7	1290 ± 250	16.1 ± 6.8
Chl ($\mu g\ cm^{-2}$)	49.0 ± 4.2		12.0 ± 2.4	

[a] Experiments performed with a different set of plants from those used for GPDHase and FBPase activities; growth conditions were identical for both groups.

min at 12,000 *g* in order to determine the inorganic Pi liberated colorimetrically (5).

RPKase activity was analyzed as described by Wolosiuk and Buchanan (25). The enzyme was activated by incubating 0.1 ml of the extract with 0.1 ml of 0.1 M Tricine (pH 7.6) and 15 mM DTT for 1 h at 25°C. After the activation was completed, 0.75 ml of the reaction medium was added. The reaction medium consisted of 66 mM Tricine (pH 7.6), 13 mM $MgCl_2$, 1.3 mM P-pyruvic acid, 1.3 mM ATP, 0.33 mM NADH, and 2 units each of phosphoriboisomerase, pyruvate kinase, and lactic dehydrogenase. The reaction was initiated by the addition of 0.05 ml of 30 mM ribose-5-P and the oxidation of NADH monitored at 340 nm.

Chemicals. All compounds used were purchased from Sigma Chemical Company with the exception of the $MgCl_2$, $CaCl_2$, and NaCl (Mallinckrodt).

RESULTS AND DISCUSSION

Determination of the influence of Fe nutrition on the activity of GPDHase, FBPase, and RPKase is complicated by the fact that these enzymes may be activated *in vivo* by products of light-generated reactions. A ferredoxin-thioredoxin activating system has been proposed (17) for the regulation of GPDHase (3, 24), RPKase (25), and FBPase (4, 5, 16). Alternatively, GPDHase and RPKase may be modulated by light-generated vicinal-dithiols (1, 7) and GPDHase has been reported to be activated by ATP or NADPH (9, 10, 15, 24). Thus, because these three enzymes require activation, it was necessary to add exogenous compounds to substitute for light activation *in vivo*. GPDHase was preincubated with ATP, and RPKase and FBPase with DTT to substitute for the ferredoxin-thioredoxin system (17) or the vicinal-dithiol system (1). These *in vitro* rates therefore represent the maximum rates obtainable with enzymes activated by nonphysiological means, and although they may reflect the amount of enzyme present, they do not indicate the extent of activation *in vivo*.

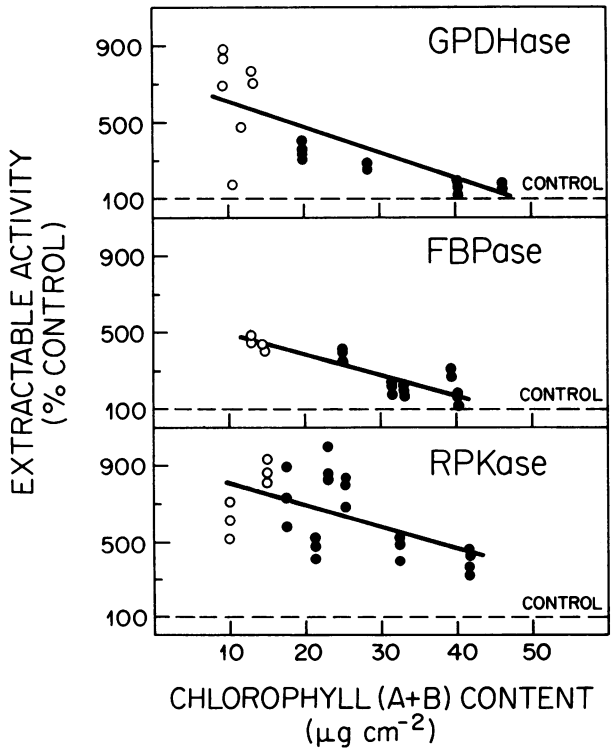

FIG. 1. Changes in GPDHase, FBPase, and RPKase activity per Chl with changes in Chl content of Fe deficient leaves. Activity is expressed as a percentage of control rate (*i.e.* rates of the enzyme extracted from plants with sufficient Fe). Plants without Fe for 7 d (○) were resupplied with iron (●) (for GPDHase, $y = -15.6x + 752$, $r = -0.785$; for FBPase, $y = -11.0x + 589$, $r = -0.874$; for RPKase, $y = -10.6x + 905$, $r = 0.519$).

The influence of Fe nutrition on the three enzymes was investigated by withholding Fe nutritionally for 7 d to induce Fe deficiency chlorosis, then resupplying Fe to regreen the plants. After 1 week without Fe, leaf Chl content decreased from 49 ± 4.2 $\mu g\ cm^{-2}$ to 12 ± 2.4 $\mu g\ cm^{-2}$. Over the same period extractable activities of the enzymes on a per Chl basis increased substantially (Table I, Fig. 1). When expressed per unit area leaf tissue, however, the activities of the three enzymes under Fe stress were only slightly elevated over those of control plants (Fig. 2).

When Fe was resupplied, the Chl content of the previously Fe-deficient leaves rapidly increased, approaching control levels in 5 d. The per area activities of GPDHase and FBPase remained above control levels and changed little throughout the regreening process. RPKase activity differed in that the activity per area increased during Fe resupply, rather than remaining constant. The activities of all three enzymes decreased on a per Chl basis during Fe resupply, which reflected mainly the increase in Chl content.

These data show that although Chl content was reduced by 76% under Fe stress, the activities of the three enzymes were not negatively affected. Although there was some variation in the activity of the three enzymes in response to Fe stress and Fe resupply, the changes were generally much smaller than the changes in Chl content. Thus, these data, along with the previously reported data on RuBP carboxylase and lamellar components (19, 23), support the conclusion that Fe stress influences photosynthesis via changes in the concentration of lamellar components rather than via changes in the enzymes of photosynthetic carbon metabolism. However, since Fe stress might influence the level of activation of the enzymes *in vivo*, we can infer only that Fe stress does not deplete the amount of enzyme present.

In reviewing the literature, it is evident that although some studies support the view that Fe stress preferentially affects lamellar components (*e.g.* Price and Carell [14]), others do not. Stocking (20) found that Fe stress reduced RuBP carboxylase activity as well as ferredoxin levels and lamellar content in corn. Perur *et al.* (12) reported an 82% reduction in chloroplast protein in iron deficient corn, whereas Shetty and Miller (18) observed a significant reduction in chloroplast protein (along with decreases in Chl and carotenoids) during Fe deficiency in tobacco. These more wide-ranging effects may be related to the duration and

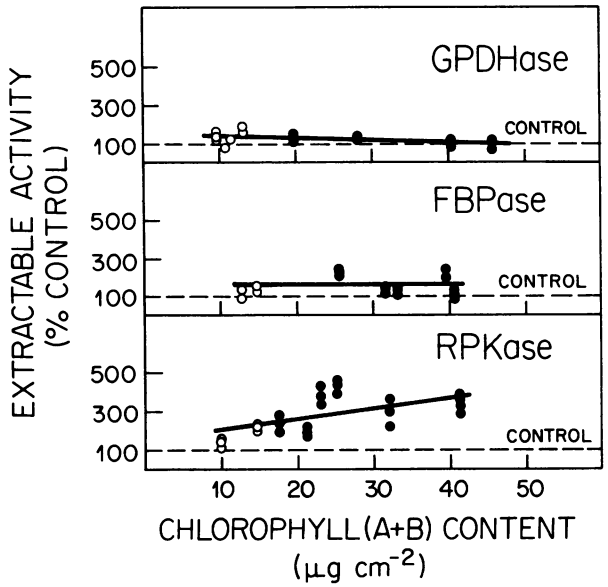

FIG. 2. Changes in GPDHase, FBPase, and RPKase activity per area with changes in Chl content of Fe deficient leaves. Activity is expressed as a percentage of control rate. Plants without Fe for 7 d (○) were resupplied with iron (●) (for GPDHase, $y = -0.901x + 143$, $r = -0.436$; for FBPase, $y = -0.042x + 151$, $r = -0.010$; for RPKase, $y = 4.91x + 167$, $r = 0.486$).

therefore severity of iron stress. Plants that are exposed to long periods of Fe stress may become damaged to the point where leaf tissue becomes necrotic, and the metabolic changes which precede necrosis could result in substantial losses in stromal proteins and/or enzymic activity.

In our laboratory, where we imposed Fe stress for periods of not more than 6 to 8 d, we found that Fe stress reduced the content of lamellar components on a per chloroplast or per leaf area basis without altering other leaf attributes. These attributes included soluble leaf protein, protein per chloroplast, the number of chloroplasts per area or per cell, the number of cells per leaf area, or mean cell volume (19, 23). Furthermore, although Fe stress caused the cessation of lamellar development, young leaves grew at a constant high rate and attained the same leaf thickness, fresh weight per area, and tissue volume as leaves of control plants at an equivalent stage of development (22). These facts suggest that the effects of short-term Fe stress on lamellar development are reasonably specific. This view is further supported by recent work of Platt-Aloia, Thomson, and Terry (13), who found that the cells of Fe stressed leaves were normal with respect to the ultrastructure of all organelles except chloroplasts: the mitochondria and microbodies, for example, which have Fe-containing complexes, were identical to the corresponding organelles in control plants.

In conclusion, we believe the present work is important because it helps establish the validity of using Fe stress as a technique to study photosynthetic limitation *in vivo*. These data extend and confirm the hypothesis that Fe stress effects on photosynthesis are mediated by changes in the lamellar components of chloroplasts rather than by changes in the content of stromal enzymes involved in photosynthetic carbon reduction. In the next paper of this series, we will present data showing how Fe deficiency-mediated changes in photochemical capacity influence photosynthetic rate in different light/CO_2 environments.

Acknowledgment—We thank Cliff Carlson for his excellent technical assistance, and Dr. Bob Buchanan for his assistance with the enzyme assays.

LITERATURE CITED

1. ANDERSON LE, M AVRON 1976 Light modulation of enzyme activity in chloroplasts. Generation of membrane-bound vicinal-dithiol groups by photosynthetic electron transport. Plant Physiol 57: 209–213
2. ARNON DI 1949 Copper enzymes in isolated chloroplasts. Polyphenoloxidase in *Beta vulgaris*. Plant Physiol 24: 1–15
3. BUCHANAN BB, NA CRAWFORD, RA WOLOSIUK 1978 Ferridoxin-thioredoxin system functions with effectors in activation of NADP-glyceraldehyde 3-phosphate dehydrogenase of barley seedlings. Plant Sci Lett 12: 257–264
4. BUCHANAN BB, PP KALBERER, DI ARNON 1967 Ferredoxin-activated fructose diphosphatase in isolated chloroplasts. Biochem Biophys Res Commun 29: 74–79
5. BUCHANAN BB, P SCHÜRMANN, PP KALBERER 1971 Ferridoxin-activated fructose diphosphate of spinach chloroplasts. Resolution of the system, properties of the alkaline fructose diphosphatase component, and physiological significance of the ferridoxin-linked activity. J Biol Chem 246:5952–5959
6. CHU DK, JA BASSHAM 1974 Activation of ribulose 1,5-diphosphate carboxylase by nicotinamide adenine dinucleotide phosphate and other chloroplast metabolites. Plant Physiol 54: 556–559
7. FISCHER KH, E LATZKO 1979 Chloroplast ribulose 5-phosphate kinase: light-mediated activation, and detection of both soluble and membrane-associated activity. Biochem Biophys Res Commun 89: 300–306
8. FLÜGGE UI, M STITT, M FREISL, HW HELDT 1982 On the participation of phosphoribulokinase in the light regulation of CO_2 fixation. Plant Physiol 69: 263–267
9. LATZKO E, M GIBBS 1968 Distribution and activity of enzymes of the reductive pentose phosphate cycle in spinach leaves and in chloroplasts isolated by different methods. Z Pflanzenphysiol 59: 184–194
10. MULLER B 1970 On the mechanism of the light-induced activation of the NADP-dependent glyceraldehyde phosphate dehydrogenase. Biochim Biophys Acta 205: 102–109
11. NISHIZAWA AN, BC YEE, BB BUCHANAN 1982 Chloroplast fructose 1,6-bisphosphatase from spinach leaves. *In* M Edelman, RB Hallick, NH Chua, eds, Methods in Chloroplast Molecular Biology, Elsevier/North Holland Press, in press
12. PERUR NG, RL SMITH, HH WIEBE 1961 Effect of iron chlorosis on protein fractions of corn leaf tissue. Plant Physiol 36: 736–739
13. PLATT-ALOIA KA, WW THOMSON, N TERRY 1982 Changes in plastid ultrastructure during iron-mediated chloroplast development. Protoplasma. In press
14. PRICE CA, EF CARELL 1964 Control by iron of chlorophyll formation and growth in *Euglena gracilis*. Plant Physiol 39: 862–867
15. PUPILLO P, GG GUILIANI-PICCARI 1973 The effect of NADP on the subunit structure and activity of spinach chloroplast glyceraldehyde-3-phosphate dehydrogenase. Arch Biochem Biophys 154: 324–331
16. SCHÜRMANN P, RA WOLOSIUK 1978 Studies on the regulatory properties of chloroplast fructose-1,6-bisphosphatase. Biochim Biophys Acta 522: 130–138
17. SCHÜRMANN P, RA WOLOSIUK, VD BREAZEALE, BB BUCHANAN 1976 Two proteins function in the regulation of photosynthetic CO_2 assimilation in chloroplasts. Nature (Lond) 263: 257–258
18. SHETTY AS, GW MILLER 1966 Influence of iron chlorosis on pigment and protein metabolism in leaves of *Nicotinamide tabacum* L. Plant Physiol 41: 415–421
19. SPILLER S, N TERRY 1980 Limiting factors in photosynthesis. II. Iron stress diminishes photochemical capacity by reducing the number of photosynthetic units. Plant Physiol 65: 121–125
20. STOCKING CR 1975 Iron deficiency and the structure and physiology of maize chloroplasts. Plant Physiol 55: 626–631
21. TERRY N, RP HUSTON 1975 Effect of calcium on the photosynthesis of intact leaves and isolated chloroplasts of sugar beets. Plant Physiol 55: 923–927
22. TERRY N 1979 The use of mineral nutrient stress in the study of limiting factors in photosynthesis. *In* R Marcelle, H Clijster, M Van Poucke, eds, Photosynthesis and Plant Development. Proceedings of Symposium, Dipenbeek-Hasselt Belgium, July 1978. Dr. W. Junk, The Hague, pp. 151–160
23. TERRY N 1980 Limiting factors in photosynthesis. I. Use of iron stress to control photochemical capacity *in vivo*. Plant Physiol 65: 114–120
24. WOLOSIUK RA, BB BUCHANAN 1976 Studies on the regulation of chloroplast NADP-linked glyceraldehyde-3-phosphate dehydrogenase. J Biol Chem 251: 6456–6461
25. WOLOSIUK RA, BB BUCHANAN 1978 Regulation of chloroplast phosphoribulokinase by the ferredoxin/thioredoxin system. Arch Biochem Biophys 189: 97–101