Ferredoxin-dependent CO_2 fixation in bean sprouts

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Abstract Extracts of bean sprouts are capable of reducing ferredoxin and of catalyzing the incorporation of bicarbonate and acetyl coenzyme A into an organic compound that is likely to be pyruvate, in a reaction that requires reduced ferredoxin. The rate of the reaction, the first known for which ferredoxin appears to serve as the direct reductant for CO_2 fixation in a higher plant, depends on the concentrations of both ferredoxin and bicarbonate, with half-maximal rates being observed at ferredoxin and bicarbonate concentrations of 0.8 μM and 200 μM, respectively.

Key words: Ferredoxin; CO_2 assimilation; Reductive carboxylation; Non-photosynthetic tissue; Bean sprout

1. Introduction

The role of ferredoxin as the terminal electron acceptor in light-driven, oxygenic photosynthesis is well established [1]. Ferredoxin reduced by photosystem I serves as the electron donor for the ferredoxin:$NADP^+$ oxidoreductase (FNR)-catalyzed reduction of $NADP^+$ [1], providing the NADPH required for the reductive conversion of CO_2 to carbohydrate, and also as the electron donor for the reduction of thioredoxin, providing the reduced thioredoxin required for the activation of several key enzymes involved in CO_2 assimilation [1,2]. Recently it has become clear that ferredoxin is present in many non-photosynthetic plant tissues, where it probably functions as an electron donor involved in nitrogen assimilation [3]. To date, no evidence has been presented for the involvement of reduced ferredoxin, as opposed to NADPH, as the immediate electron donor for CO_2 assimilation in any oxygenic photosynthetic organism, although the role of ferredoxin-dependent reductive carboxylations in CO_2 assimilation during anoxygenic photosynthesis has been well documented [4]. Recent work in our laboratory has demonstrated the presence of ferredoxin, FNR and two low-potential cytochromes in bean sprouts, a non-photosynthetic tissue [5–7]. Below, as part of our continuing characterization of the role of ferredoxin in bean sprouts, we present evidence that suggests that bean sprouts possess the enzymatic machinery necessary for catalyzing a ferredoxin-dependent carboxylation of acetyl CoA.

2. Materials and methods

Bean sprouts (chlorophyll free) and spinach leaves were purchased from local markets. Bean sprout ferredoxin ($A_{421\ nm}/A_{277\ nm} = 0.45$), spinach leaf ferredoxin ($A_{422\ nm}/A_{277\ nm} = 0.45$) and spinach leaf nitrite reductase were prepared as described previously [5,8,9] and stored at liquid nitrogen temperature. Acetyl CoA, succinyl CoA, β-mercaptoethanol, 2,4-dinitrophenylhydrazine, phenylmethylsulfonyl fluoride (PMSF), *t*-amylalcohol, sodium pyruvate, bovine serum albumin, Sigmacell Type 100 cellulose polyester thin layer chromatography sheets, and [^{14}C]sodium bicarbonate were purchased from Sigma Chemical Co. [^{14}C]acetyl CoA was purchased from ICN. No. 2 filter paper was purchased from Whatman. Benzyl isothiocyanate (BITC) was purchased from Aldrich.

Absorbance spectra and time-courses were measured using a Shimadzu Model UV2100U spectrophotometer. Protein concentrations were measured according to the method of Bradford [10], using bovine serum albumin as a standard. Nitrite reductase concentrations were calculated from protein concentrations, using a value of $M_r = 63$ kDa for the enzyme [1]. Concentrations of oxidized spinach leaf ferredoxin [11], oxidized bean sprout ferredoxin [5], and reduced methyl viologen [12], were determined using extinction coefficients of 9.7 $mM^{-1} \cdot cm^{-1}$ at 422 nm, 8.9 $mM^{-1} \cdot cm^{-1}$ at 421 nm and 14.0 $mM^{-1} \cdot cm^{-1}$ at 604 nm, respectively.

Nitrite reductase activity was measured as previously described [13]. Ferredoxin reduction and ferredoxin-stimulated oxidation of reduced methyl viologen, catalyzed by bean sprout extracts, were measured at 25°C in a 1 cm optical pathlength, Thunberg-style cuvette. Reaction mixtures were made anaerobic by repeated cycles of evacuation and flushing with helium. Methyl viologen was reduced by granular metallic zinc and was introduced into the cuvette anaerobically using an apparatus similar to that described by Asada [12]. Ferredoxin-dependent oxidation of reduced methyl viologen was initiated by tipping in acetyl CoA from the side arm of the Thunberg cuvette. The 2,4-dinitrophenylhydrazone derivatives were prepared according to the method of Rabinowitz [14], and were analyzed by either paper chromatography on Whatman No. 2 paper or by thin layer chromatography on cellulose polyester sheets using a 5:1:4 mixture of *t*-amylalcohol/ethanol/water as the mobile phase [15]. Radioactivity was measured using Beckman Model LS-7000 and LS-5000TD liquid scintillation counters.

Crude bean sprout homogenates were prepared by grinding the sprouts at 4°C in 250 mM potassium phosphate buffer (pH 7.7) with a mortar and pestle, and filtering the homogenate through cheese cloth. Total protein concentrations of these homogenates ranged from 1.8 to 5.4 mg/ml. Bean sprout homogenates used for acetone fractionation were prepared by homogenizing 500 g of bean sprouts in a Waring blender for 1 min at 4°C in 475 ml of 100 mM Tris-HCl buffer (pH 8.0) containing 400 mM NaCl, 0.1% β-mercaptoethanol, 0.5 mM EDTA, 0.1 mM BITC and 1 mM PMSF. The homogenate was filtered through cheese cloth and chilled acetone was added. The fraction precipitating between 35% and 75% acetone/water (v/v) was collected by centrifugation at $10,000 \times g$ for 5 min at −20°C and resuspended in a minimum volume of the homogenizing buffer. After dialysis against the homogenizing buffer, followed by dialysis against 10 mM Tris-HCl buffer (pH 8.0) containing 200 mM NaCl and 0.1% β-mercaptoethanol, the sample was centrifuged to remove insoluble materials and the protein concentration of the supernatant was adjusted to 35 mg protein/ml.

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Abbreviations: BITC, benzyl isothiocyanate; EDTA, ethylenediamine tetraacetic acid; FNR, ferredoxin:$NADP^+$ oxidoreductase; PMSF, phenylmethylsulfonyl fluoride.

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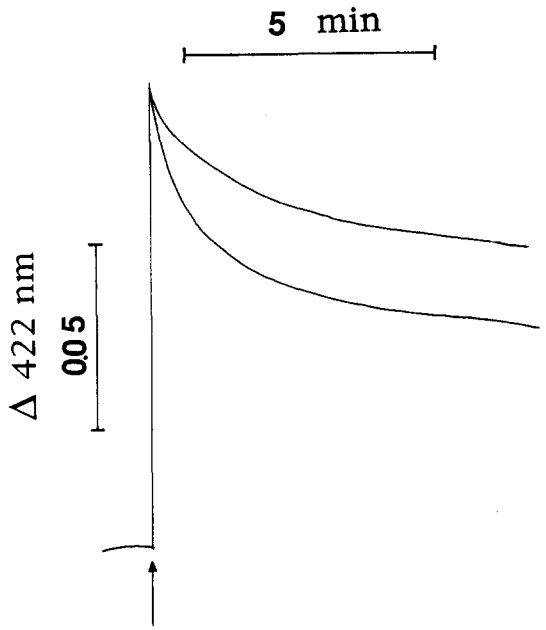

Fig. 1. Reduction of ferredoxin by a bean sprout homogenate. The reaction mixture contained 54 μM spinach leaf ferredoxin in 30 mM Tris-HCl buffer (pH 8.0) under a He atmosphere. The upper trace represents an experiment in which 0.09 mg of crude bean sprout homogenate protein was present and the lower trace represents an experiment in which 0.18 mg of crude bean sprout homogenate protein was present. The experiment was initiated by addition of the homogenate and the vertical offset was adjusted so that the pen positions after the initial rapid absorbance increases due to the addition of the colored homogenate were identical for both experiments.

3. Results

Using decreases in the absorbance at 422 nm to monitor ferredoxin reduction, it was possible to demonstrate that a crude homogenate of bean sprouts is capable of reducing spinach leaf ferredoxin (Fig. 1). Increasing the amount of bean sprout extract increased the rate of ferredoxin reduction (Fig. 1). Similar results were obtained with bean sprout ferredoxin (data not shown). If nitrite *plus* spinach leaf nitrite reductase (an enzyme that is highly specific for reduced ferredoxin among physiological electron donors) were added to the reaction mixture, reduction of nitrite could be measured, confirming that the absorbance decrease at 422 nm represented a net reduction of ferredoxin. No nitrite reduction was observed if either the crude extract, nitrite reductase or ferredoxin were omitted, or if either methyl viologen, NAD^+, or $NADP^+$ replaced ferredoxin. The identity of the endogenous reductant for ferredoxin in the crude bean sprout extract is not yet known.

Table 1 demonstrates that the crude bean sprout homogenate is capable of catalyzing the incorporation of bicarbonate into an organic compound. The two-fold stimulation in CO_2 incorporation observed when ferredoxin is added, suggests that ferredoxin is the likely electron donor for the observed CO_2 fixation. It was not necessary to add an exogenous reductant, as the crude bean sprout homogenate was capable of reducing ferredoxin itself (see Fig. 1). The activity observed in the absence of added ferredoxin may arise either from the presence of endogenous ferredoxin, which is known to be present in the crude homogenate [5], or from a separate ferredoxin-independent pathway. In contrast to the stimulation observed when ferredoxin was added, no stimulation of CO_2 fixation was observed when either NADH, FMN or FAD was added to the reaction mixture, either in the presence or absence of added ferredoxin. A pattern similar to that shown in Table 1 was observed for the formation of a radioactive product precipitable by 2,4-dinitrophenylhydrazine if [^{14}C]acetyl CoA was added to the reaction mixture and [^{12}C]bicarbonate was present instead of [^{14}C]bicarbonate (data not shown).

Addition of NADPH to reaction mixtures to which exogenous ferredoxin was added produced no additional stimulation in the extent of CO_2 incorporation, but did stimulate CO_2 incorporation in the absence of added ferredoxin. The stimulation of CO_2 fixation produced by NADPH observed with this extract may result from the presence of enzymes not related directly to the carboxylation reaction. For example, the crude extract is known to contain FNR [6], which can catalyze the reduction of ferredoxin by NADPH [6]. However, it is not clear why the stimulation of CO_2 fixation produced by the addition of NADPH was not observed in the presence of exogenous ferredoxin.

Addition of ATP, in the presence of either Mg^{2+} or Mn^{2+}, produced no stimulation of CO_2 fixation (data not shown). Thus the CO_2 fixation observed, which appears to show an absolute requirement for a reductant, can not arise from a reaction catalyzed by an ATP-dependent acetyl CoA carboxylase, a reductant-independent enzyme known to be present in some plant tissues [16].

Results similar to those of Table 1 were observed with a 35–75% acetone-precipitated fraction of the bean sprout homogenate (prepared as described in section 2). Using this acetone fraction it could be demonstrated that addition of acetyl CoA produced an approximately 50% increase in the amount of radioactivity incorporated, and that succinyl CoA could not replace acetyl CoA in producing this stimulation (data not shown).

The rate of CO_2 fixation catalyzed by the bean sprout homogenate depended on ferredoxin concentration, as would be expected for a reaction in which ferredoxin served as the immediate electron donor. Double-reciprocal plots of the rate vs. ferredoxin concentration were linear, with an apparent K_m for ferredoxin equal to 0.8 μM. Double-reciprocal plots of the rate

Table 1
CO_2 fixation catalyzed by a bean sprout homogenate

	Radioactivity of the 2,4-dinitrophenylhydrazone precipitate (cpm)
Complete	11,174
− ferredoxin	5,792
− $^{14}CO_2$	32
− bean sprout homogenate	741

The 3.0 ml reaction mixture contained the bean sprout homogenate (5.37 mg protein), 1 mg of spinach leaf ferredoxin and 40 μM $NaCHO_3$ (specific activity = 6.5 mCi/mmol) in 250 mM potassium phosphate buffer (pH 7.7). The reaction was carried out under a He atmosphere, but the reaction mixture was not rigorously degassed. The reaction was initiated by addition of the crude bean sprout homogenate and was stopped, after a 30 min incubation, by addition of 2.0 ml of a saturated 2,4-dinitrophenylhydrazine solution in 2 M HCl. The experimental errors in the radioactivity of the samples were ca. ±20%.

Table 2
Ferredoxin oxidation catalyzed by an acetone-fractionated bean sprout extract

	Rate of methyl viologen oxidation (% of control)
Complete	100
−acetyl CoA	4.6
−ferredoxin	44.5
−sodium bicarbonate	7.2
−bean sprout extract	5.8

The 3.0 ml reaction mixture present in the main compartment of a Thunberg cuvette contained 0.5 mg of spinach leaf ferredoxin, the acetone-fractionated bean sprout homogenate (equivalent to 14 mg of protein), 3 mg of bovine serum albumin, 2.0 μmol of sodium bicarbonate and 2.0 μmol of reduced methyl viologen in 50 mM HEPES buffer (pH 7.5) under a He atmosphere. The reaction was initated by tipping in 3.1 μmol of acetyl CoA in a volume of 0.1 ml.

of the reaction as a function of bicarbonate concentration were not linear. Half-maximal rates were observed at a bicarbonate concentration of 200 μM.

If bean sprouts can catalyze carboxylation of acetyl CoA, with reduced ferredoxin serving as the direct electron donor, then one should be able to observe an oxidation of reduced ferredoxin catalyzed by a bean sprout homogenate when bicarbonate and acetyl CoA are present. The high background absorbance at 422 nm of the acetone-fractionated bean sprout homogenate, which had a higher absorbance than the crude extract used for the experiment shown in Fig. 1, made it difficult to follow changes in the oxidation state of ferredoxin directly. However, as reduced methyl viologen rapidly reduces ferredoxin, it is possible to follow the oxidation of reduced ferredoxin indirectly by monitoring absorbance decreases resulting from the ferredoxin-dependent oxidation of reduced methyl viologen at 604 nm, a wavelength where interference from the background absorbance of the extract is less severe. Table 2 summarizes the results of such an experiment in which the oxidation of reduced methyl viologen is initiated by tipping in acetyl CoA from the side arm of an anaerobic Thunberg cuvette. Addition of spinach leaf ferredoxin produces a greater than two-fold increase in the rate of methyl viologen oxidation and the oxidation is almost completely dependent on the presence of both bicarbonate and acetyl CoA. Acetyl CoA could not be replaced by succinyl CoA (data not shown). Bean sprout ferredoxin was as effective as spinach leaf ferredoxin in stimulating the bicarbonate/acetyl CoA-dependent oxidation of reduced methyl viologen, but the addition of either NAD^+ or $NADP^+$ had no effect on the rate of oxidation of reduced methyl viologen (data not shown).

The observation that the radioactive compound formed from [^{14}C]bicarbonate was precipitable with 2,4-dinitrophenylhydrazine indicates that the reaction product contains a carbonyl group. The observation that acetyl CoA was also incorporated into the precipitable product made it seem likely that the product was pyruvate. Indeed, during chromatography of the ethylacetate-extracted 2,4-dinitrophenylhydrazone derivative of the product (using a 5:1:4 mixture of *t*-amylalcohol/ethanol/water as the mobile phase), the radioactive material migrated as a single spot with the same R_f value as that of the 2,4-dinitrophenylhydrazone derivative of an authentic pyruvate standard when either Whatman No. 2 paper or cellulose polyester thin layer sheets were used as the stationary phase.

4. Discussion

The observations that the addition of ferredoxin can stimulate the incorporation, catalyzed by a bean sprout homogenate, of bicarbonate and acetyl CoA into an organic compound, and that the homogenate can oxidize reduced ferredoxin in a reaction that requires the presence of both bicarbonate and acetyl CoA, are both consistent with the presence in bean sprouts of the enzymatic machinery needed to catalyze a reductive carboxylation of acetyl CoA with ferredoxin acting as the direct electron donor. The fact that the product appears to be pyruvate, the product expected from a reductive carboxylation of acetyl CoA, provides additional support for the hypothesis. Despite the apparent instability of at least one of the components involved in the carboxylation reaction, attempts are currently underway in our laboratory to purify the enzyme(s) involved in catalyzing this CO_2 fixation reaction, a reaction that is novel in plant tissues. In addition to opening the way for mechanistic studies of the enzyme(s) involved, purification of the bean sprout homogenate will hopefully make it possible to obtain sufficient amounts of the product of the CO_2 fixation reaction to unambiguously identify it.

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