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Comparative amino acid sequence of fructose-1,6-bisphosphatases: Identification of a region unique to the light-regulated chloroplast enzyme

(light-dependent activation/photosynthesis)

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ABSTRACT **Chloroplast fructose-1,6-bisphosphatase (Fru-P_2-ase) is an essential enzyme in the photosynthetic pathway of carbon dioxide fixation into sugars. The properties of the chloroplast enzyme are clearly distinct from cytosolic gluconeogenic Fru-P_2-ases. Light-dependent activation by way of a ferredoxin/thioredoxin system and insensitivity to AMP inhibition are distinctive characteristics of the chloroplast enzyme. However, the chloroplast enzyme shows a high degree of amino acid sequence similarity to gluconeogenic Fru-P_2-ases. Sequence data reported for a total of 285 residues (≈75% of the structure) of the spinach chloroplast enzyme reveals a 46% amino acid sequence identity with pig kidney Fru-P_2-ase. We now report the amino acid sequence of a region consisting of 46 additional residues. This region is located near the middle of the primary structure of the enzyme and it includes a 16-residue insert not present in other Fru-P_2-ases. This sequence insert has two cysteines separated by only 4 amino acid residues (Cys-Val-Val-Asn-Val-Cys), a characteristic feature of at least three other enzymes containing redox-active cysteines. It appears likely that this region of chloroplast Fru-P_2-ase is involved in light-dependent activation.**

Light plays an important role in the regulation of several enzymes involved in the synthetic or carbon-reduction phase of photosynthesis and in related biochemical processes. In most cases, light produces the activation of several chloroplast enzymes that are essentially inactive in the dark. The *in vivo* regulation of all these enzymes involves the ferredoxin/thioredoxin system, which comprises ferredoxin, thioredoxin *f* or *m*, and ferredoxin/thioredoxin reductase. One of these light-regulated enzymes is chloroplast fructose-1,6-bisphosphatase (Fru-P_2-ase), the enzyme that catalyzes the conversion of fructose 1,6-bisphosphate to fructose 6-phosphate. The light-mediated activation Fru-P_2-ase occurs *in vivo* by way of a ferredoxin/thioredoxin *f* system that converts an inactive oxidized form of the enzyme into reduced active Fru-P_2-ase (see refs. 1 and 2). The activation process can be mimicked *in vitro* by reduction of the enzyme with dithiothreitol (3). In both cases, the activation reaction involves the reduction of a critical disulfide bond in the enzyme. This activation mechanism is a characteristic of chloroplast Fru-P_2-ase that distinguishes the chloroplast enzyme from cytosolic Fru-P_2-ase (4, 5). In addition, the chloroplast enzyme is not sensitive to AMP inhibition (6, 7), a property of all cytosolic gluconeogenic Fru-P_2-ases (4). Nevertheless, the chloroplast enzyme shows a high degree of amino acid sequence similarity with gluconeogenic Fru-P_2-ases that suggests a common evolutionary origin for all Fru-P_2-ases in spite of their different functions and modes of regulation (8, 9). Amino acid sequence data reported for a total of 285 residues (≈75% of the structure) of the spinach chloroplast enzyme (9, 10) reveals a 46% sequence identity with pig kidney Fru-P_2-ase. These studies have disclosed the position of three cysteine residues of spinach chloroplast Fru-P_2-ase, but these cysteine residues are probably not those involved in the light regulation of the enzyme (9). We have now identified a segment of the protein molecule that shows no similarity in sequence to that of other Fru-P_2-ases and that is located near the middle of the primary structure of the enzyme. This region contains two cysteines that are separated by only 4 amino acid residues and is likely to be involved in light-dependent activation of chloroplast Fru-P_2-ase.

MATERIALS AND METHODS

Spinach chloroplast Fru-P_2-ase was purified to homogeneity as described by Marcus *et al.* (10). This enzyme is herein referred to as oxidized chloroplast Fru-P_2-ase. Enzyme activity was determined as described (10), except that the assays were performed at pH 8.5 as well as at pH 8.0. In each case, the assay contained 50 mM Tris·HCl at the above stated pH values. The activity of the purified enzyme measured at pH 8.5 was 65–80 units/mg but exhibited an activity of only 5 units/mg when the assay was performed at pH 8.0. Higher enzyme activity at pH 8.0 is a characteristic of reduced chloroplast Fru-P_2-ase, and this enzyme form can be prepared *in vitro* by reduction of oxidized chloroplast Fru-P_2-ase with dithiothreitol (3, 7, 11). For this purpose, purified chloroplast Fru-P_2-ase was dialyzed against a buffer containing 50 mM Hepes·NaOH (pH 7.5), 0.1 M NaCl, and 0.2 mM EDTA and then incubated at a concentration of 20–50 μg/ml with 3 mM $MgSO_4$ and 25 mM dithiothreitol. After 16 hr at 22°C, the activity of the enzyme measured at pH 8.0 increased to 60–80 units/mg as the result of the formation of reduced enzyme.

The reduced chloroplast Fru-P_2-ase was incubated with 55 mM 4-vinylpyridine (Aldrich) to achieve a 30 mM excess over the concentration of dithiothreitol. After 90 min at 22°C, the reaction mixture was dialyzed against two 500-ml changes of 3% (vol/vol) formic acid. The pyridylethylated protein was then digested for 24 hr with pepsin at a 500:1 (wt/wt) ratio of Fru-P_2-ase to pepsin. Under these experimental conditions, pepsin appears to cleave specifically at the carboxyl side of leucine and phenylalanine residues (12). The reaction products of pepsin treatment were separated by reversed-phase HPLC as described (13). The column effluent was monitored at 214 nm, as well as at 254 nm, which provides a sensitive identification of pyridylethylcysteine-containing peptides (14). Control experiments were performed as described

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Abbreviation: Fru-P_2-ase, fructose-1,6-bisphosphatase.

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above, but reduction of Fru-P_2-ase with dithiothreitol was omitted and pyridylethylation with 49 mM vinylpyridine was carried out with oxidized enzyme unfolded in 6 mM guanidine hydrochloride.

The techniques used for *S*-carboxymethylation and cleavage of oxidized chloroplast Fru-P_2-ase with cyanogen bromide were those described (13), except that after lyophilization the dried mixture of CNBr fragments was suspended in 2 ml of 30% (vol/vol) CH_3COOH. The soluble fraction was separated by centrifugation and the pellet was washed twice with 30% CH_3COOH. The insoluble fraction was dissolved in 0.5 ml of 0.1 M potassium phosphate/1 M urea, pH 7.8, and treated for 16 hr at 22°C with *Staphylococcus aureus* V8 protease (Miles). Under these conditions, cleavage of peptide bonds occurs only at the carboxyl side of glutamic acid residues (15). The reaction products were separated by reversed-phase HPLC as described (13).

Subtilisin treatment and *S*-carboxymethylation of chloroplast Fru-P_2-ase, as well as the tryptic digestion of the *S*-carboxymethylated protein, was performed as described by Marcus *et al.* (10). The tryptic peptides were separated by reversed-phase HPLC as described (figure 2 of ref. 10). Automated microsequencing of peptides (0.2–1.5 nmol) was performed in an Applied Biosystems (Foster City, CA) model 470A gas-phase protein sequencer using the standard sequencing program and the reagents provided by the manufacturer. The phenylthiohydantoin amino acid derivatives, liberated after each degradation cycle, were identified and quantitated as such by HPLC with a modification of the procedure described by Hunkapiller (16). The analyses were performed with a Waters Associates system model 840 equipped with an Altex (Berkeley, CA) Ultrasphere ODS 5-μm column (4.6 × 250 mm). Amino acid analyses were performed as described by Bidlingmeyer *et al.* (17). Peptides (100–500 pmol) were hydrolyzed in vapor of 6 M HCl for 22 hr at 110°C. The liberated amino acids were incubated with phenyl isothiocyanate, and the resulting derivatives were analyzed by reversed-phase HPLC on a Waters system model 840 equipped with a Waters Pico-Tag column.

RESULTS AND DISCUSSION

The amino acid sequence of a 46-residue segment of spinach chloroplast Fru-P_2-ase was assembled by aligning the sequences of the peptide fragments herein designated as P14, CB2-5, and TS-4a. Peptide P14 (Fig. 1*A*) was obtained by pepsin digestion of reduced pyridylethylated chloroplast Fru-P_2-ase. We selected this peptide for sequence analysis for two reasons. First, the peptide was rich in cysteine residues as deduced from the high absorbance of P14 at 254 nm (data not shown). Second, peptide P14 was nearly absent in the HPLC pattern of the control experiments. In these control experiments the reduction of Fru-P_2-ase with dithiothreitol was omitted and pyridylethylation was carried out with the enzyme unfolded in 6 M guanidine hydrochloride. Thus, it was inferred that P14 was a peptide containing cysteine(s) that originated from a dithiothreitol-reduced disulfide bond in the enzyme. Automated Edman degradation of peptide P14 gave the sequence Ser-Ala-Glu-Glu-Gln-Arg-PEC-Val-Val-Asn-Val-PEC-Gln-Pro-Gly-Asp-Asn-Leu, where PEC is *S*-β-(4-pyridylethyl)cysteine. This amino acid sequence aligned with a region ending in Leu-159 of pig kidney Fru-P_2-ase (18), but the overall similarity was of only 27% (5 identities in 18 residues). Remarkably, however, the 5 amino acid position identities were all located in the COOH-terminal sequence Gln-Pro-Gly-Asp-Asn-Leu of P14. Thus, this terminal hexapeptide revealed a very strong homology (83%), while the first 12 residues of the sequence showed a much lower similarity (33%) with a sequence of pig kidney Fru-P_2-ase. It was also noted that peptide P14 contained an unusual sequence consisting of two cysteine residues separated by only 4 amino acid residues, a feature shown by several enzymes containing redox-active cysteines (19) but not found in the characterized Fru-P_2-ases (18, 20,

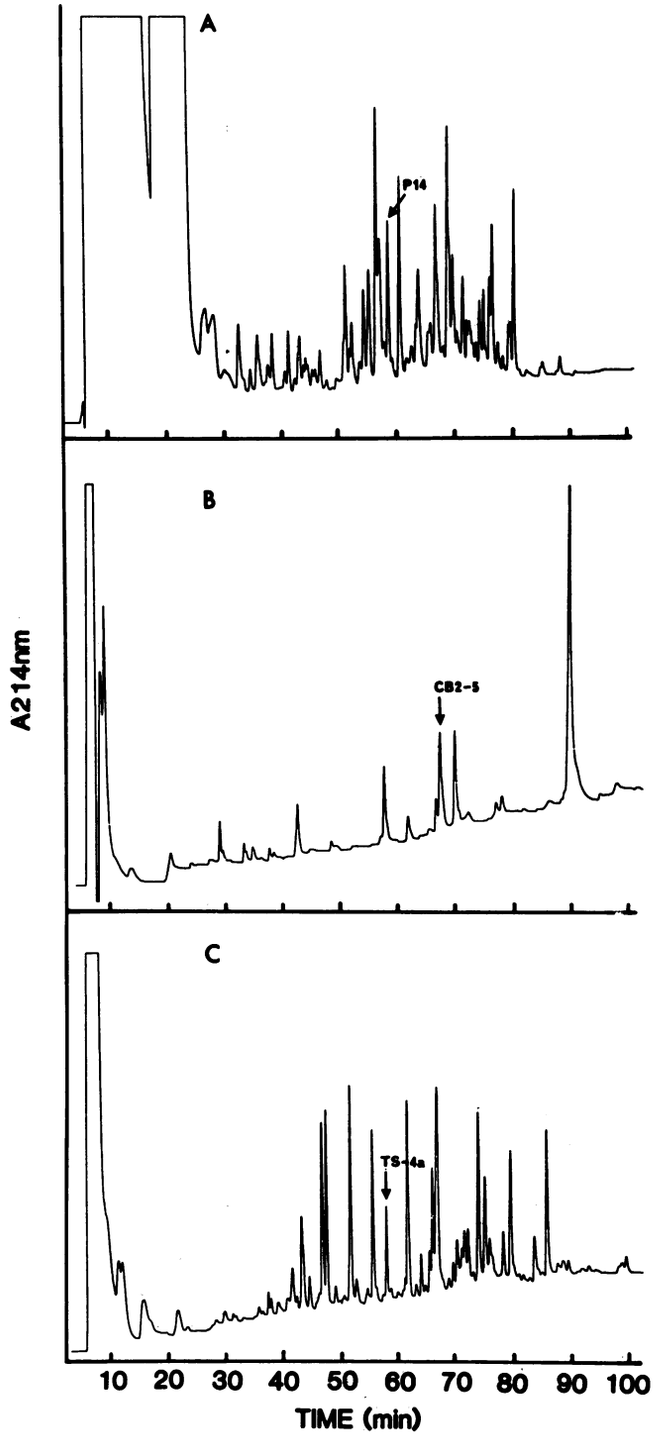

FIG. 1. Reversed-phase HPLC of a peptic digest of *S*-pyridylethylated reduced spinach chloroplast Fru-P_2-ase (*A*); a *S. aureus* protease digest of the largest cyanogen bromide fragment of *S*-carboxymethylated oxidized spinach chloroplast Fru-P_2-ase (*B*); and a tryptic digest of subtilisin-treated *S*-carboxymethylated oxidized spinach chloroplast Fru-P_2-ase (*C*). Samples were acidified to pH 4 with 30% (vol/vol) CH_3COOH, and 1–2 nmol was injected into a Bio-Rad RP-304 column (4.6 × 250 mm) equilibrated with 0.1% trifluoroacetic acid. Peptides were eluted with a 0–45% (vol/vol) acetonitrile gradient containing 0.1% trifluoroacetic acid over 90 min with a flow rate of 0.5 ml/min. The peaks shown with arrows and designated as noted were selected for amino acid sequence analysis.

Table 1. Amino acid sequence analysis of selected peptide fragments of spinach chloroplast Fru-P_2-ase

Cycle number	Amino acid residue		
	Peptide P14	Peptide CB2-5	Peptide TS-4a
1	Ser (133)	Gln (509)	Gly (168)
2	Ala (254)	Arg (51)	Ile (154)
3	Glu (198)	CmC (242)	Tyr (110)
4	Glu (198)	Val (392)	Ser (45)
5	Gln (195)	Val (437)	Pro (126)
6	Arg (79)	Asn (286)	Asn (71)
7	PEC (73)	Val (359)	Asp (65)
8	Val (155)	CmC (219)	Glu (94)
9	Val (158)	Gln (425)	CmC (58)
10	Asn (109)	Pro (366)	Ile (72)
11	Val (110)	Gly (284)	Val (57)
12	PEC (48)	Asp (197)	Asp (54)
13	Gln (87)	Asn (254)	Ser (29)
14	Pro (96)	Leu (291)	Asp (50)
15	Gly (65)	Leu (336)	His (18)
16	Asp (43)	Ala (271)	Asp (40)
17	Asn (46)	Ala (334)	Asp (58)
18	Leu (21)	Gly (220)	Glu (21)
19		Tyr (201)	Ser (10)
20		CmC (108)	Gln (19)
21		Hse (41)	Leu (14)
22			Ser (9)
23			Ala (11)
24			Glu (11)
25			Glu (17)
26			Gln (12)
27			Arg (2)
Number of residues*	168–185	172–192	147–173

Amino acid sequences of peptides, designated as shown in Fig. 1, were determined. Recoveries in pmol are given in parentheses. PEC, pyridylethylcysteine; CmC, *S*-carboxymethylcysteine; Hse, homoserine.

*Provisional numbers are based on our current knowledge of the primary structure of spinach chloroplast Fru-P_2-ase and its homology with other Fru-P_2-ases.

21). These sequence characteristics led us to search for additional peptides to provide overlaps and extensions of the sequence, since it appeared that we had found an area that was rather specific to the chloroplast enzyme.

The second fragment that provided additional sequence information was peptide CB2-5. This peptide had its origin in CB2, an acid-soluble 161-residue CNBr fragment beginning at a position equivalent to Ala-24 of pig kidney Fru-P_2-ase (sequence results not shown). Treatment of *S*-carboxymethylated CB2, with *S. aureus* protease resulted in the formation of several peptides that were isolated and were subjected to sequence analysis. Peptide CB2-5 (Fig. 1*B*), a peptide containing three carboxymethylcysteine residues, confirmed the sequence of residues 5–18 of P14 and added 7 residues (Table 1). The additional residues included a tetrapeptide Ala-Ala-Gly-Tyr, a sequence also found in pig kidney Fru-P_2-ase (residues 161–164) and thus leaving no doubt with regard to the location of the sequenced peptides.

The NH_2-terminal extension of the sequenced region was obtained by analysis of TS-4a, a peptide (Fig. 1*C*) obtained from a tryptic digest of subtilisin-treated *S*-carboxymethylated chloroplast Fru-P_2-ase. It appears that treatment of chloroplast Fru-P_2-ase with subtilisin results in major cleavage at the subtilisin-sensitive region of Fru-P_2-ases (10), plus minor proteolytic cleavage near the middle of the chloroplast Fru-P_2-ase molecule. This secondary cleavage plus the action of trypsin resulted in the formation of the 27-residue peptide TS-4a that was sequenced in its entirety (Table 1). The first 21 residues of peptide TS-4a (glycine through leucine) provided additional sequence information, while cycles 22–27 corresponded to sequenced portions of P14 and CB2-5.

Based on our current knowledge of the primary structure of spinach chloroplast Fru-P_2-ase and on the homology of the chloroplast enzyme with other Fru-P_2-ases, we tentatively locate the structures disclosed in Table 1 in a segment spanning the sequence of residues 147–192 of spinach chloroplast Fru-P_2-ase. This sequence is shown in Fig. 2 aligned together with the sequence of the so far known Fru-P_2-ases. This alignment clearly demonstrates that spinach chloroplast Fru-P_2-ase has amino acid identities with the other Fru-P_2-ases (shown by dots above the chloroplast Fru-P_2-ase sequence) that permit the alignment, but it is also obvious that chloroplast Fru-P_2-ase contains an insert of ≈15 amino acids that is specific to this enzyme. Contained within the insert are two cysteines separated by only 4 other amino acid residues, a feature shown by at least three other enzymes containing redox-active cysteines (19). Most notably, an 8-residue sequence of chloroplast Fru-P_2-ase can be aligned with the active site sequence of mercuric reductase, glutathione reductase, and lipoamide dehydrogenase (Fig. 3). The similarities are evident. In all four enzymes, the two cysteines are separated by 4 amino acids, the dipeptide Asn-Val is located between the two cysteines, and a proline is located 2 residues upstream of the second cysteine. The redox-active thiols of two ribonucleotide reductases (24) have also been located in a

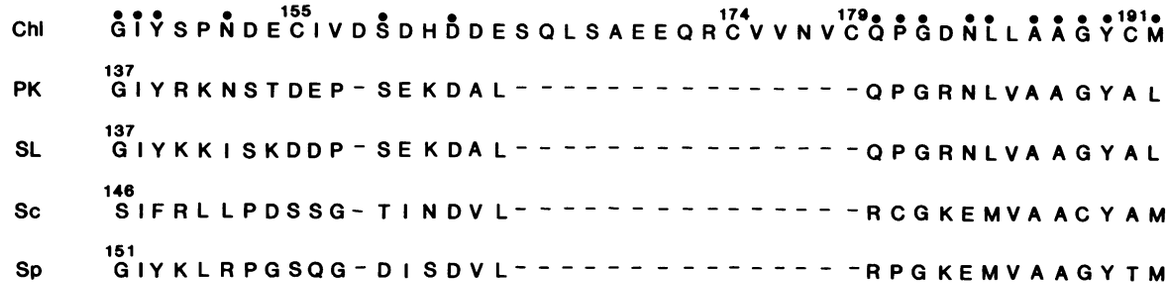

FIG. 2. Alignment and comparison of the sequenced region of spinach chloroplast Fru-P_2-ase with the amino acid sequence of other Fru-P_2-ases. Amino acids are indicated by the single-letter code. The sequenced region of spinach chloroplast Fru-P_2-ase (Chl) comes from the data of Table 1. The sequence data of pig kidney (PK), sheep liver (SL), and the two yeasts (*Saccharomyces cerevisiae*, Sc; and *Schizosaccharomyces pombe*, Sp) Fru-P_2-ases are taken from Marcus *et al.* (18), Fisher and Thompson (20), and Rogers *et al.* (21), respectively. In these four sequences, the number above the first residue indicates its location in the corresponding amino acid sequence and a deletion in position 12 has been introduced for better alignment. A dot above some spinach chloroplast Fru-P_2-ase amino acids indicates that this residue is also found in at least one of the other four Fru-P_2-ases. These common residues are the basis for the alignment and reveal a sequence insert present only in the chloroplast enzyme. This insert is shown above a row of dashes in the other four Fru-P_2-ases. The numbers above the cysteine residues of spinach chloroplast Fru-P_2-ase indicate provisional locations based on our current knowledge (published data of refs. 9 and 10 plus unpublished results) of the amino acid sequence.

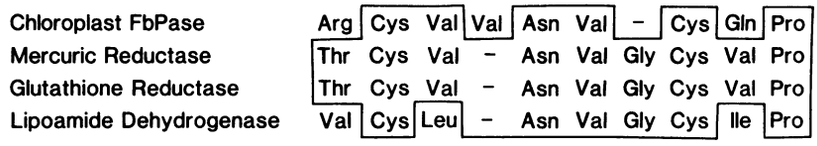

FIG. 3. Comparison of amino acid sequences in the region of the spatially close cysteines of spinach chloroplast Fru-P_2-ase (FbPase) and those at the active site of mercuric reductase and of two nicotinamide disulfide oxidoreductases. The sequences shown for *Pseudomonas aeruginosa* mercuric reductase, human erythrocyte glutathione reductase, and *Escherichia coli* lipoamide dehydrogenase are from Fox and Walsh (19), Untucht-Grau *et al.* (22), and Burleigh and Williams (23), respectively. A box is used to highlight amino acid residue identities between the four sequences. Some insertions, denoted by dashes, have been introduced to maximize sequence homology.

sequence of amino acids containing two cysteines separated by 4 other residues. Pea chloroplast NADP-malate dehydrogenase, another light-regulated enzyme, also contains two cysteine residues separated by 4 other residues, but it is not known yet whether these residues, which are not found in NAD-malate dehydrogenases, are those involved in light-dependent regulation (25). In other examples (thioredoxin, glutaredoxin, protein disulfide isomerase, and thioredoxin reductase; refs. 26–28), the redox-active thiols are also spatially close since they are separated by only 2 amino acid residues. The best known example is thioredoxins that show a conserved structure Cys-Gly-Pro-Cys, which, in the oxidized form consists of a 14-member disulfide ring located on a protrusion exposed to the solvent (29).

Table 2. Comparison of amino acid sequences around positions equivalent to the cysteine residues of spinach chloroplast Fru-P_2-ase

Source of Fru-P_2-ase	Sequence	Ref.
	51	
Chloroplast	Ser–Leu–Ala–Cys–Lys–Gln–Ile	This work*
Pig kidney	Cys–Thr–Ala–Val–Lys–Ala–Ile	18
Sheep liver	Cys–Thr–Ala–Val–Lys–Ala–Ile	20
Sa. cerevisiae	Gln–Phe–Ala–Phe–Lys–Phe–Val	21
Sc. pombe	Gln–Phe–Ser–Phe–Lys–Phe–Ile	21
	94	
Chloroplast	Phe–Ser–Ser–Cys–Leu–Arg–Ser	9
Pig kidney	Val–Ile–Asn–Val–Leu–Lys–Ser	18
Sheep liver	Val–Val–Asn–Val–Leu–Lys–Ser	20
Sa. cerevisiae	Phe–Ile–Asn–Ala–Met–Arg–Ala	21
Sc. pombe	Phe–Ile–Thr–Ala–Met–Lys–Ser	21
	155	
Chloroplast	Asn–Asp–Glu–Cys–Ile–Val–Asp	9
Pig kidney	Asn–Ser–Thr–Asp–Glu–Pro–Ser	18
Sheep liver	Ile–Ser–Lys–Asp–Asp–Pro–Ser	20
Sa. cerevisiae	Leu–Pro–Asp–Ser–Ser–Gly–Thr	21
Sc. pombe	Arg–Pro–Gly–Ser–Gln–Gly–Asp	21
	174 179	
Chloroplast	Arg–Cys–Val–Val–Asn–Val–Cys	This work
Pig kidney	NE	18
Sheep liver	NE	20
Sa. cerevisiae	NE	21
Sc. pombe	NE	21
	191	
Chloroplast	Ala–Gly–Tyr–Cys–Met–Tyr	This work
Pig kidney	Ala–Gly–Tyr–Ala–Leu–Tyr	18
Sheep liver	Ala–Gly–Tyr–Ala–Leu–Tyr	20
Sa. cerevisiae	Ala–Cys–Tyr–Ala–Met–Tyr	21
Sc. pombe	Ala–Gly–Tyr–Thr–Met–Tyr	21
	310	
Chloroplast	Leu–Tyr–Glu–Cys–Ala–Pro–Met	9
Pig kidney	Leu–Tyr–Glu–Cys–Asn–Pro–Met	18
Sheep liver	Leu–Tyr–Glu–Cys–Asp–Pro–Met	20
Sa. cerevisiae	Leu–Tyr–Glu–Ala–Phe–Pro–Met	21
Sc. pombe	Leu–Tyr–Glu–Cys–Phe–Pro–Met	21

Numbers above the cysteine residues indicate provisional locations based on our current knowledge (published data of refs. 9 and 10, plus unpublished results) of the amino acid sequence of spinach chloroplast Fru-P_2-ase. NE, nonexistent; *Sa. cerevisiae*, *Saccharomyces cerevisiae*; *Sc. pombe*, *Schizosaccharomyces pombe*.
*This sequence was determined by automated Edman degradation of CB2-5 and the sequence shown corresponds to cycles 16 through 22.

The present work also completes the determination of the amino acid sequences around the seven cysteine residues of spinach chloroplast Fru-P_2-ase. The determined sequences are shown in Table 2 and compared with corresponding sequences in the four entirely sequenced Fru-P_2-ases. Except for the sequence containing the two cysteine residues likely to be involved in light-dependent regulation of chloroplast Fru-P_2-ase, all other cysteine-containing sequences can be aligned with known Fru-P_2-ase structures. The comparison demonstrates, however, that six of the seven cysteine residues of spinach chloroplast Fru-P_2-ase are not found in the other Fru-P_2-ases. This observation indicates that these six cysteine residues play no essential general role in either enzyme structure or catalysis. The seventh cysteine (Cys-310), which is conserved in all sequences except *S. cerevisiae* Fru-P_2-ase, is located in a highly conserved region that appears to be involved in substrate binding (30). It is also noteworthy that the cysteine-containing sequences of spinach chloroplast Fru-P_2-ase show no significant similarities to either the sequences surrounding Cys-16 and -54 of spinach ribulose 5-phosphate kinase (31) or to the cysteine-containing sequences of pea chloroplast NADP-malate dehydrogenase (25), suggesting that light-dependent regulation in these three plant enzymes evolved independently.

As shown in Fig. 2, the determined 46-residue sequence of chloroplast Fru-P_2-ase can be aligned under a region of the sequence of pig kidney Fru-P_2-ase that begins with residue 137 and ends with residue 167. Although the three-dimensional structure of pig kidney Fru-P_2-ase is not yet known (32), a model postulated from secondary structure predictions (33) may be pertinent to the present findings. In this model, pig kidney Fru-P_2-ase is viewed as having two domains. The first domain is formed by residues 1–141, a second domain contains residues 157–335; and both domains are joined by a 17-residue exposed strand of random-coiled structure. It is precisely in this region, postulated to join the two domains, where the specific chloroplast Fru-P_2-ase insert is located.

Note. Since the submission of this paper, we have found that the specific 16-residue insert of the spinach chloroplast Fru-P_2-ase is not present in the spinach cytosolic enzyme (U. Ladror and F.M., unpublished results).

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