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SEQUENCES OF TWO ACTIVE-SITE PEPTIDES FROM SPINACH RIBULOSEBISPHOSPHATE CARBOXYLASE/OXYGENASE*

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SUMMARY

Two tryptic peptides from spinach ribulosebisphosphate carboxylase/oxygenase that contain the essential lysyl residues derivatized by the affinity label 3-bromo-1,4-dihydroxy-2-butanone 1,4-bisphosphate were subjected to sequence analyses. The sequences of these peptides are -Tyr-Gly-Arg-Pro-Leu-Leu-Gly-Cys-Thr-Ile-Lys-Pro-Lys- and -Leu-Ser-Gly-Gly-Asp-His-Ile-His-Ser-Gly-Thr-Val-Val-Gly-Lys-Leu-Glu-Gly-Glu-Arg-, respectively. The reagent moiety is covalently attached to the internal lysyl residue in each peptide.

Despite the direct dependence of plant life on Rbl-P_2[†] carboxylase/oxygenase (E. C. 4.1.1.39) (the enzyme required for the photosynthetic fixation of CO_2) and thus the indirect dependence of animal life on the enzyme, its structure and mechanism of action remain rather poorly characterized (see refs. 1 and 2 for reviews). With the realization that the enzyme has inherent oxygenase activity and can thus account for photorespiration (3,4), a metabolic process which decreases net CO_2 fixation (see ref. 5 for a review), the elucidation of structural-functional relationships acquires an added dimension of significance.

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[†]Abbreviations: Rbl-P_2, D-ribulose 1,5-bisphosphate; Br-butanone-P_2, 3-bromo-1,4-dihydroxy-2-butanone 1,4-bisphosphate.

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The carboxylase/oxygenase from higher plants (e.g. spinach) has a molecular weight of 560,000 and contains eight protomeric units, each composed of a large (56,000 daltons) and small (14,000 daltons) subunit (6-8). In view of this molecular complexity, affinity labeling would appear to offer the best opportunity for mapping the active site of Rbl-P_2 carboxylase/oxygenase. With Br-butanone-P_2 as an affinity label, inactivation of the spinach enzyme correlates with the selective alkylation of two lysyl residues (9). A variety of data are consistent with these lysyl residues being in the active-site region, including the observation that the analogous enzyme from the pylogenetically distant organism R. rubrum is also inactivated by the affinity label as a consequence of lysyl modification (10). In this communication, we report amino acid sequences of the two tryptic peptides that contain the essential lysyl residues present in the spinach enzyme. To our knowledge, these sequences are the first determined for regions encompassing a portion of the active site of any Rbl-P_2 carboxylase/oxygenase.

MATERIALS AND METHODS

The peptides to be subjected to sequence analyses were purified from tryptic digests of Rbl-P_2 carboxylase/oxygenase after inactivation by Br-butanone-P_2 followed by reduction with [^{3}H]$NaBH_4$ and carboxymethylation with iodoacetic acid according to the published procedure (9). Reduction with [^{3}H]borohydride converts the carbonyl of the covalently-bound reagent moiety to a hydroxyl with concomitant incorporation of tritium at the carbon atom originally bearing the carbonyl oxygen. Each of the two labeled peptides purified contains a derivatized lysyl residue; inactivation of the enzyme by Br-butanone-P_2 correlates with modification of these two lysyl residues, and Rbl-P_2 protects them against modification (9). In the previous study, the two peptides (designated I and II) were shown to be composed of (derivatized-Lys_1, Lys_1, Arg_1, Cm-Cys_1, Thr_1, Pro_2, Gly_2, Ile_1, Leu_2, Tyr_1) and (derivatized-Lys_1, His_2, Arg_1, Asp_1, Thr_1, Ser_2, Glu_2, Gly_5, Val_2, Ile_1, Leu_2), respectively. Amino acid compositions of peptides I and II isolated for the present sequence work are the same as those published.

All chemicals (benzene, 1-chlorobutane, n-heptafluorobutyric acid, phenyl isothiocyanate, and N,N-dimethyl-N-allylamine) required for Edman degradations were purchased from Pierce Chem. Co. Carboxypeptidases A and B that had been treated with diisopropylfluorophosphate were products of Sigma Chem. Co.

Edman degradations were carried out on 50-120 nmol of peptide in a Beckman 890C sequencer with peptide program #102974 provided by Beckman. Anilinothiazolinones were identified as free amino acids after base (11) or acid (12) hydrolysis. Amino acid analyses were run on a Beckman 121M analyzer with the single-column system as described by the manufacturer. Asp and Glu

were distinguished from their corresponding amides by conversion of the anilinothiazolinones to the phenylthiohydantoins which were then identified by thin-layer chromatography (13).

Standard procedures were used for carboxypeptidase digestions (14) and for quantitation of radioactivity by scintillation spectrometry (15).

RESULTS AND DISCUSSION

The data obtained from automated Edman degradations on peptides I and II are presented in Table I. These experiments were repeated twice without discrepancies arising. By the use of both base and acid hydrolysis, as well as thin-layer chromatography for confirmation, the identification of the amino acid anilinothiazolinone released at each cycle is unequivocal with two exceptions — gaps are seen at cycle 11 in peptide I and cycle 15 in peptide II, presumably representing the derivatized lysyl residues. This assumption is confirmed by elevated levels of radioactivity in the fractions obtained at these two cycles (Table I). The carry-over of radioactivity into subsequent fractions is easily explained, since the derivatized lysyl residue (which bears a phosphate moiety) should be difficult to extract with chlorobutane (the solvent used to extract the anilinothiazolinone at each cycle). In the case of peptide I, significant radioactivity is also seen at cycles 3-5. This probably reflects an increased solubility in chlorobutane of the peptide fragment remaining after the polar arginyl residue is released at cycle 3; the presence of peptide in the chlorobutane extract has been confirmed by analyses of total acid hydrolysates. The partially degraded peptide then becomes less soluble in organic solvents after removal of the leucyl residues at positions 5 and 6.

The following sequences are thus established (the asterisk indicates the modified residues):

(peptide I) -Tyr-Gly-Arg-Pro-Leu-Leu-Gly-Cys(Cm)-Thr-Ile-Lys*-Pro-Lys-

(peptide II) -Leu-Ser-Gly-Gly-Asp-His-Ile-His-Ser-Gly-Thr-Val-Val-Gly-Lys*-Leu-Glu-Gly-Glu-Arg-

Digestion of peptides I and II with mixtures of carboxypeptidases A and B liberated only Lys (1 molar equivalent) from the former and only Arg (1 molar

TABLE 1

Edman Degradation of Labeled Peptides

Cycle	Peptide I			Peptide II		
	Amino Acid	Yield (%)	Radioactivity*	Amino Acid	Yield (%)	Radioactivity*
1	Tyr	94	1.2	Leu	97	1.6
2	Gly	85	2.2	Ser††	-	1.7
3	Arg†	44	8.0	Gly	92	1.9
4	Pro	43	6.5	Gly	76	1.5
5	Leu	30	2.2	Asp	69	1.8
6	Leu	34	1.5	His	37	1.7
7	Gly	24	1.3	Ile	65	1.7
8	Cys(Cm)**	8	1.5	His	36	1.6
9	Thr	6	2.3	Ser††	-	1.3
10	Ile	19	1.8	Gly	29	1.2
11	-	-	9.7	Thr	27	1.1
12	Pro	15	7.9	Val	46	1.0
13	Lys	3	7.6	Val	41	1.1
14				Gly	35	3.1
15				-	-	10.1
16				Leu	19	6.2
17				Glu	22	4.4
18				Gly	22	3.4
19				Glu	18	2.7
20				Arg†	9	1.9

*Radioactivity is expressed as percentage of total present in peptide preceding cycle 1.

†Identification of Arg is based on presence of ornithine in base hydrolysates.

**Identification of Cys(Cm) is based on presence of alanine in both acid and base hydrolysates.

††Identification of Ser is based on presence of alanine in acid hydrolysates and on thin-layer chromatography of the phenylthiohydantoin.

equivalent) from the latter. These results are consistent with the above sequences and known specificities of the carboxypeptidases (14).

The presence of prolyl residues in peptide I at positions 4 and 12 excludes the possibility of this segment being derived from a lengthy α-helical stretch in the native enzyme. More interesting is the rather high preponderance

of basic residues in this peptide (Arg-3, Lys-11, Lys-13, and Arg or Lys preceding Tyr-1, since the peptide is a product of tryptic digestion). A region containing a cluster of positive charges is certainly consistent with electrostatic requirements for a binding site that accommodates a bisphosphate. Noteworthy features of peptide II are not obvious.

The availability of sequences from the active-site region of Rbl-P_2 carboxylase/oxygenase will permit interesting comparisons as additional peptides that contain residues implicated as active-site components are isolated. For example, pyridoxal phosphate also inactivates the enzyme by a selective modification of lysyl residues (16). If a lysyl residue modified proves to be one of the same as labeled by Br-butanone-P_2 (i.e. those present in peptide I and II), additional evidence for active-site modification and functional significance of a given lysyl residue will have been provided. Whether the inactivation of the carboxylase/oxygenase by -SH reagents (for a discussion see ref. 17) involves modification of the cysteinyl residue found in peptide I can also be determined. Finally, in view of the frequency at which arginyl residues are involved in binding of phosphoesters (18), attempts to determine if the arginyl residue of peptides I or II is crucial to substrate binding would appear warranted.

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