Ribulosebisphosphate Carboxylase: Amino Acid Sequence of a Peptide Bearing the Activator Carbon Dioxide†

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ABSTRACT: Ribulosebisphosphate carboxylase is activated by reaction of an activator CO_2 to form a carbamate on the ϵ-amino group of a lysyl residue on the large catalytic subunit. This carbamate has been converted to the methoxycarbonyl derivative by treatment of the enzyme with diazomethane as previously reported [Lorimer, G. H., & Miziorko, H. H. (1980) *Biochemistry 19*, 5321]. Digestion of the methylated enzyme–$^{14}CO_2$ complex with trypsin yielded several radioactive peptides which were purified by using standard chromatographic procedures. Sequence analyses revealed that these peptides had the same sequence: -Gly-Gly-Leu-Asp-Phe^5-Thr-Lys-Asp-Asp-Glu^{10}-Asn-Val-Asn-Ser-Gln^{15}-Pro-Phe. Residue 7 was ^{14}C labeled and emerged from the sequencer as the phenylthiohydantoin derivative of N^{ϵ}-(methoxycarbonyl)lysine. The acidic nature of the residues close to the lysine bearing the activator CO_2 provides a molecular explanation for the pH and divalent metal ion dependency of the activation reaction. An entirely homologous sequence has been found in the large subunit of the enzyme from *Zea mais* [McIntosh, L., Poulsen, C., & Bogorad, L. (1980) *Nature (London) 288*, 556]. The lysyl residue bearing the activator CO_2 is 26 residues removed from one of the lysyl residues identified by use of the affinity label *N*-bromoacetylethanolamine phosphate as being within the active-site domain.

Ribulose-1,5-bisphosphate[1] carboxylase (EC 4.1.1.39) is activated by the reaction of CO_2 with the ϵ-amino group of a lysyl residue located on the large catalytic subunit (Lorimer et al., 1976; O'Leary et al., 1979; Lorimer & Miziorko, 1980). The molecule of CO_2 involved in the activation reaction (carbamate formation) is distinct from that which becomes fixed during catalysis (Miziorko, 1979; Lorimer, 1979).

In a previous report, we described a highly specific method for stabilizing the enzyme carbamate in a form capable of withstanding the rigors of protein chemistry (Lorimer & Miziorko, 1980). Briefly, the activator carbamate was esterified by treatment with diazomethane.

$$\sim(CH_2)_4NH_2 + {}^{A}CO_2 \underset{}{\overset{\pm H^+}{\rightleftharpoons}}$$
$$\sim(CH_2)_4NH^{A}COO^- \xrightarrow{CH_2N_2} \sim(CH_2)_4NH^{A}COOCH_3$$

The product of this reaction was identified as N^{ϵ}-(methoxycarbonyl)lysine (Lorimer & Miziorko, 1980).

Two lysyl residues within the active-site domain have been identified by affinity labeling techniques (Hartman et al., 1978). The sequence of amino acids surrounding these critical lysyl residues has been determined (Hartman et al., 1978). With the identification of a large subunit lysine as the site of activator carbamate formation, it became imperative to determine the sequence of the adjacent amino acids. In this report, the sequence of the peptide bearing the activator CO_2 is revealed to be different from those peptides bearing the active-site lysyl residues.

Experimental Procedures

Materials

RuBP carboxylase was purified from spinach leaves, activated, and assayed as previously described (Lorimer et al., 1976, 1977). Enzyme protomer was determined at 280 nm by using an extinction coefficient of 1.15×10^5 M^{-1} cm^{-1} (Paulsen & Lane, 1966). Diazomethane[2] was generated from *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (Aldrich Chemical Co.) as previously described (Lorimer & Miziorko, 1980). The synthesis of authentic N^{ϵ}-(methoxycarbonyl)lysine was conducted as before (Lorimer & Miziorko, 1980). CABP was prepared according to the procedure of Pierce et al. (1980).

The following materials were obtained from the suppliers listed in parentheses: RuBP (Sigma Chemical Co.); $Na_2{}^{14}CO_3$ (Amersham); Sephadex and DEAE-Sephacel (Pharmacia Fine Chemicals); ion-exchange resins (Bio-Rad Labs); trypsin ostensibly treated with L-(tosylamido-2-phenyl)ethyl chloromethyl ketone (Worthington Biochemical Corp.).

Methods

Preparation of the Methylated Enzyme·Activator $^{14}CO_2$ Complex. Three micromoles (210 mg) of the enzyme·activator $^{14}CO_2$·Mg·CABP complex ($^{14}CO_2$/protomer = 1.06; ^{14}C specific activity = 9840 dpm/nmol) was prepared and separated from the unbound $^{14}CO_2$ by gel filtration chromatography vs. 50 mM Bicine, 20 mM $NaHCO_3$, and 20 mM $MgCl_2$, pH 8.2, as previously described (Lorimer & Miziorko, 1980). This complex was treated with diazomethane by using the same procedure as before (Lorimer & Miziorko, 1980) (yield of methylated enzyme·activator $^{14}CO_2$ = 80%; activator $^{14}CO_2$/methylated protomer = 0.41).

Tryptic Digestion of the Methylated Enzyme·Activator $^{14}CO_2$ Complex. The insoluble methylated enzyme·activator $^{14}CO_2$ complex was recovered by centrifugation and uniformly suspended with 10 mL of 0.20 M Tris-HCl, pH 8.2, containing 1 mM $CaCl_2$. Trypsin (10 mg) was added, and the suspension was incubated at 37 °C for about 24 h. The turbid suspension became noticeably clearer as the digestion progressed. However, an insoluble core comprising about 10% of the total ^{14}C radioactivity resisted digestion. Ice-cold water (50 mL) was added, and the insoluble material was removed by centrifugation. The supernatant solution was stored at −35 °C until ready for further processing. Unless otherwise specified, the

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[1] Abbreviations used: RuBP, ribulose bisphosphate; CABP, 2-carboxyarabinitol bisphosphate; Bicine, *N,N'*-bis(2-hydroxyethyl)glycine.

[2] CAUTION: *N*-Methyl-*N'*-nitro-*N*-nitrosoguanidine is a very potent mutagen and should be handled with the greatest care. In addition, diazomethane is not only highly toxic but also volatile and explosive. The procedure used here should only be conducted in a well-ventilated hood behind a safety shield.

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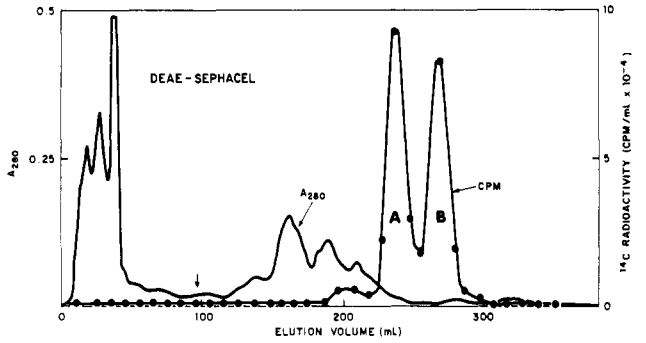


FIGURE 1: Ion-exchange chromatography on DEAE-Sephacel of the hydrolyzate obtained from the tryptic digestion of the methylated enzyme activator $^{14}CO_2$ complex. Details under Methods.

subsequent manipulations were conducted at room temperature.

Purification of the Methylated Peptides Bearing the Activator $^{14}CO_2$. The tryptic digest was first applied to a 1.6 × 16 cm column of DEAE-Sephacel equilibrated with 0.02 M NH_4HCO_3, pH 8.15. The column was next thoroughly washed with this buffer, and a substantial quantity of unlabeled 280 nm absorbing material was eluted. A linear 500-mL gradient (0.02–0.10 M NH_4HCO_3, pH 8.15) was then applied. About 80% of the ^{14}C radioactivity applied to the column emerged in the form of two peaks of approximately equal size, narrowly separated from one another (Figure 1). These peaks were designated "peptide A" and "peptide B" in the order of their elution. Both peptides were individually subjected to further purification. They were rechromatographed on DEAE-Sephacel as before to ensure that they ran true (i.e., that peptide A did not give rise to peptide B or vice versa) and to ensure that the two peptides were entirely resolved from one another. Each peak of ^{14}C radioactivity was pooled and lyophilized to remove the NH_4HCO_3.

The residue from each peak was dissolved in a small volume of water and applied to a 2.6 × 90 cm column of Sephadex G-25 (fine) equilibrated at 4 °C with 1% (v/v) formic acid. In both cases a single peak of ^{14}C radioactivity emerged well behind the void volume but in advance of the totally included material. The molecular weight of both peptides was estimated to be about 2000 on this basis. The peak fractions were pooled and lyophilized. The residue was taken up with a small volume of water and the pH adjusted to 2.9 with acetic acid. The sample was then applied to a 1.5 × 15 cm column of Dowex 50W-X2 (200–400 mesh) equilibrated with 0.020 M pyridine–acetic acid, pH 2.91. Peptide A was slightly retarded and was eluted from the column as a single peak of ^{14}C radioactivity with this buffer (data not shown). The elution of peptide B required the application of a 400-mL linear concentration gradient (0.020–0.20 M pyridine–acetic acid, pH 2.91). Only one peak of ^{14}C radioactivity was observed (data not shown). The overall recovery of ^{14}C radioactivity at this stage was about 50%, which approximately equal amounts of ^{14}C radioactivity in peptide A and peptide B.

Both peptides were then lyophilized. The residues were dissolved in a small volume of water and subjected to a second round of gel filtration chromatography on Sephadex G-25 as before. The radioactive fractions of each peptide were individually pooled and lyophilized.

At this stage, both peptides were subjected to sequence analyses. The results indicated that both samples were contaminated by a variable quantity of a second peptide. In the case of peptide A, the contaminating peptide constituted about 10% of the total. The same contaminating peptide was also present in peptide B, but in this case it constituted about 35%

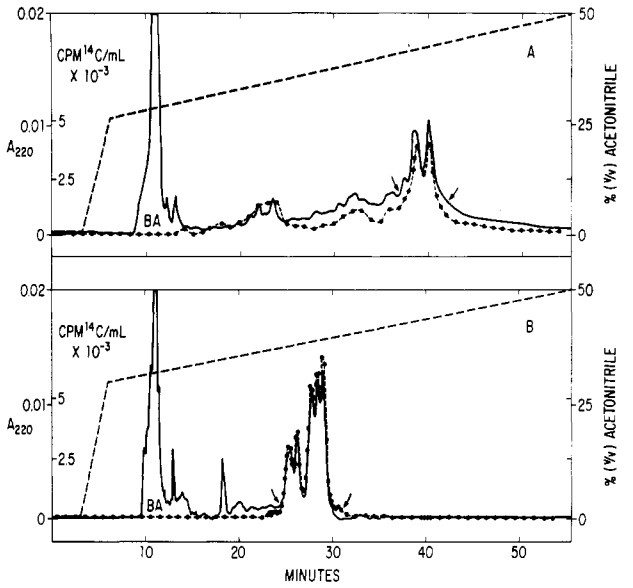


FIGURE 2: High-pressure liquid chromatography of the activator $^{14}CO_2$ peptides A and B. The arrows indicate the fractions pooled for sequence analysis. The peak marked BA is a buffer artifact. Details under Methods.

of the total. Nevertheless, two points emerged from this analysis. First, although peptides A and B exhibited different chromatographic properties, their amino acid sequences were the same. Second, the amino acid composition of the contaminating peptide (inferred from the sequence analysis) was rather similar to that of peptide A and peptide B. For separation the contaminating peptide, samples of peptide A and peptide B were subjected to reverse-phase high-pressure liquid chromatography on a 0.45 × 24 cm column packed with Du Pont Zorbax ODS and equilibrated with 0.20 M H_3PO_4, pH 2.4. The column was developed with the H_3PO_4/acetonitrile gradient system indicated in Figure 2.

Peptide B yielded one nonradioactive peak and a cluster of five radioactive peaks which contained more than 95% of the total ^{14}C radioactivity applied to the column. Collection of 10-s fractions showed that the ^{14}C radioactivity tracked with the 220-nm absorbance throughout the peaks and troughs of this cluster, indicating constant radiospecific activity. In view of this and the results of the previous sequence analysis which had indicated that this sample contained only two peptides, it was decided to combine all five peaks for subsequent amino acid composition and sequence analysis.

The reverse-phase chromatography of peptide A was less satisfactory, and considerable heterogeneity was evident even though the sample was known to contain only two peptides. Substantial amounts of 220-nm absorbing material and ^{14}C radioactivity were eluted from about 20 min on in a series of small poorly resolved peaks (Figure 2). However, two larger peaks containing about 40% of the total ^{14}C radioactivity applied to the column emerged at about 40 min. Both peaks were combined for subsequent analyses.

Several runs of both peptides were performed to accumulate sufficient purified material. Following lyophilization, each sample was again run through the Sephadex G-25 column so as to remove H_3PO_4. The ^{14}C radioactivity was pooled and lyophilized. The residues was shipped to Dr. Michael Hunkapiller of the California Institute of Technology for automated sequence analyses.

Automated Sequence Analysis. The peptides were sequenced on a modified Beckman 120B sequencer as described by Hunkapiller and Hood (1978, 1980). The phenylthiohydantoin amino acids were identified by high-pressure liquid

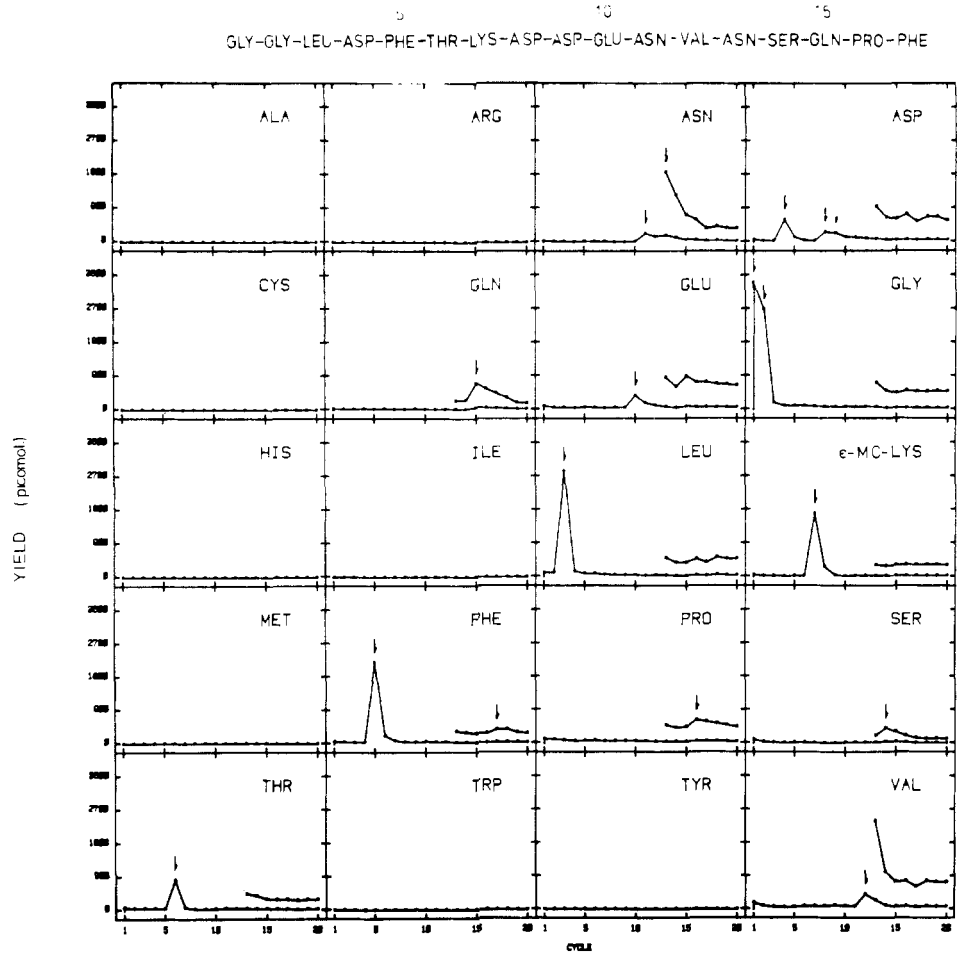


FIGURE 3: Amino acid sequence of the activator $^{14}CO_2$ peptide. Yields of phenylthiohydantoin amino acids from an N-terminal sequencer analysis of 1.0 nmol of activator $^{14}CO_2$ peptide A. Aliquots of each cycle were analyzed by high-pressure liquid chromatography. Peak heights were converted to picomoles of each derivative by using values for standard mixtures of amino acid phenylthiohydantoins, and the yields were normalized to a 100% injection.

chromatography on cyanopropylsilane columns of Zorbax (Johnson et al., 1979). The amino acid composition of the peptides was determined with hydrolyzed samples (6 N HCl, 110 °C, 24 h).

^{14}C Radioactivity Measurements. These were performed by liquid scintillation counting using the cross channel ratio method.

Results and Discussion

Purity of the Peptides. There is every reason to believe that the peptides used in the final sequence analyses were of sufficient purity to give reliable results, despite the chromatographic heterogeneity. Thus, although peptide B elutes from the high-pressure liquid chromatograph column as a cluster of five peaks, the constant relationship between the 220-nm absorbance and the ^{14}C radioactivity indicates that only one peptide was present, albeit in five slightly different guises. Also, there was satisfying agreement between the total amino acid compositions of peptide A and of peptide B determined on the acid hydrolyzate and that inferred from the sequence (Table I). Each cycle of the sequencer released essentially one residue (Figure 3). If impurities had been present, more than one residue per cycle would have been released. Finally, the specific ^{14}C radioactivity of the residue bearing the activator $^{14}CO_2$ was calculated to be 13 500 dpm/nmol, a value slightly higher than the specific radioactivity of the $^{14}CO_2$ used to label the enzyme.

The Sequence of the Activator CO_2 Peptide. The sequence of the activator CO_2 peptide (determined on peptide A) is given in Figure 3. The results are presented by plotting nanomoles of phenylthiohydantoin amino acids vs. residue position for each amino acid. The ^{14}C radioactivity of each residue released from the sequencer was also determined. Only one (residue 7) was radioactive (Figure 4). This residue did not cochro-

Table I: Amino Acid Composition of the Activator CO_2 Peptides

	peptide A		peptide B	
amino acid	from hydrolysate[a]	from sequencer	from hydrolysate[a]	from sequencer
Asp	4.7	3	4.8	3
Asn		2		2
Thr	1.1	1	1.0	1
Ser	1.1	1	1.0	1
Glu	2.0	1	2.3	1
Gln		1		1
Pro	1.1	1	1.0	1
Gly	2.0	2	2.3	2
Ala	0.09	0	0.14	0
Cys	0	0	0	0
Val	1.0	1	1.0	1
Met	0	0	0	0
Ile	0.03	0	0.07	0
Leu	1.0	1	1.0	1
Tyr	0	0	0	0
Phe	2.0	2	1.9	2
His	0.03	0	0.04	0
Lys	1.1	1[b]	1.0	1[b]
Arg	0.03	0	0.04	0
Trp	0	0	0	0

[a] Moles of amino acid/mole of peptide. [b] As N^{ϵ}-(methoxycarbonyl)lysine.

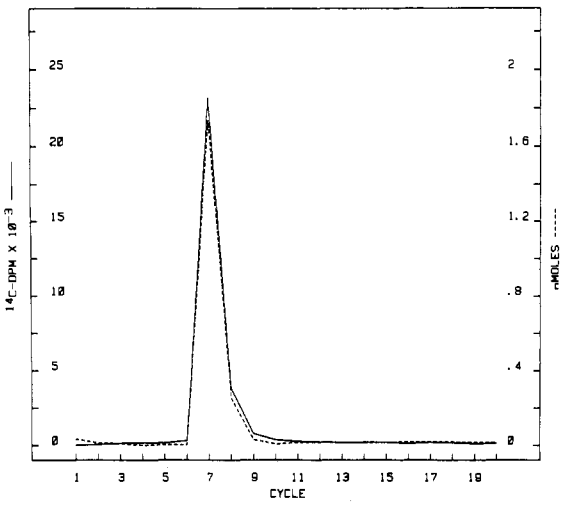


FIGURE 4: Position of the residue bearing the activator $^{14}CO_2$. Aliquots of each cycle were counted for ^{14}C radioactivity and analyzed by high-pressure liquid chromatography. Peak heights were converted to nanomoles of the phenylthiohydantoin derivative of N^{ϵ}-(methoxycarbonyl)lysine by using a standard prepared from the authentic compound. Yields were normalized to a 100% injection. The specific ^{14}C radioactivity of residue 7 was determined to be 1.35×10^4 dpm $nmol^{-1}$, a value slightly in excess of that for the $^{14}CO_2$ used to activate the enzyme initially (0.98×10^4 dpm $nmol^{-1}$).

matograph with any of the standard amino acids. We have previously identified this residue as N^{ϵ}-(methoxycarbonyl)lysine (Lorimer & Miziorko, 1980). The phenylthiohydantoin derivative of authentic N^{ϵ}-(methoxycarbonyl)lysine had the same chromatographic properties as the compound released in cycle 7 (data not shown).

Sequence analysis was also performed on peptide B. The results of this analysis were identical with those obtained for peptide A. Thus, despite their different chromatographic properties, peptides A and B have the same amino acid composition and sequence.

The nature of the C-terminal residue (Phe) deserves comment. Although the trypsin used to digest the methylated enzyme·activator·$^{14}CO_2$ complex was ostensibly treated with an inhibitor of chymotrypsin activity, it is quite evident that the activator CO_2 peptide has been cleaved at the C-terminal end by chymotrypsin.

Chromatographic Heterogeneity. Consideration of the procedure by which the carbamate was stabilized provides an explanation for the chromatographic heterogeneity that became evident during the purification of the activator CO_2 peptides. While the treatment of the enzyme with diazomethane successfully esterified the activator carbamate, it would have been surprising if this were the only residue esterified. One would expect that both glutamate and aspartate would also become esterified, although there is no guarantee that these side reactions go to completion. In the case of the activator CO_2 peptide which contains four acidic residues, as many as 16 different molecular species could be formed, ranging from the one species in which none of the acidic residues were esterified through the four monoester species, the six diesterspecies, and the four triester species to the one species in which all four residues were esterified. Clearly, the extent to which the peptide is esterified will influence its chromatographic properties. For example, peptide B was more negatively charged than peptide A and was therefore more tightly bound to the DEAE-Sephacel column (Figure 1). Peptide B might therefore consist of a mixture of the unesterified and singly esterified species while peptide A might be a mixture of doubly and triply esterified species. Reverse-phase high-pressure chromatography presumably resolved peptide B into its five constituent species (Figure 2). A similar explanation accounts for the multiple peaks observed with peptide A.

Structure–Function Relationships of the Activator CO_2 Peptide. The most striking structural feature of the activator CO_2 peptide is the distribution of the acidic residues, aspartate and glutamate. Note that the three residues next to the critical lysine are acidic, with a fourth acidic residue only three removed on the N-terminal side. It is interesting to reflect upon the possible consequences of such a structure in relation to the mechanism of activation.

Jencks and his colleagues have shown that the cleavage of carbamates of basic aliphatic amines proceeds by a mechanism involving N-protonation in a fast equilibrium step followed by CO_2 expulsion in the rate-determining step (Ewing et al., 1980). The coordination of a divalent metal ion with the carbamate would be expected to further retard this rate-limiting step.

The activation of RuBP carboxylase involves the formation of a carbamate on the ϵ-amino group of a lysyl residue, a process which brings about the release of two protons.

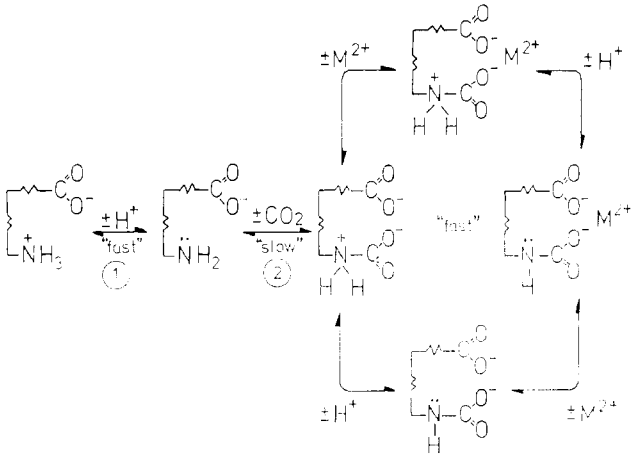
$$\sim\text{Lys-NH}_3^+ \overset{\pm H^+}{\rightleftharpoons} \sim\text{Lys-NH}_2 +$$

$$^{A}CO_2 \overset{\pm H^+}{\rightleftharpoons} \sim\text{Lys-NH-}^{A}\text{COO}^-$$

The close proximity of acidic residues is likely to influence the equilibria associated with this process. For example, one might anticipate that the pK of the ϵ-amino group would be increased above its free solution value. This would be in accord with the kinetic data (Lorimer et al., 1976) which suggests that the activator CO_2 reacts with a group of "distinctly alkaline pK". The close proximity of the acidic residues would also be expected to influence the equilibrium of carbamate formation per se, for this effectively introduces additional negative charge into an already anionic region. Thus, the equilibrium is likely to be displaced to the left in the direction of dissociation of the carbamate unless some other mechanism exists for dissipating this negative charge. The kinetics of activation indicated that the divalent metal ion displaced the equilibrium for activation to the right, i.e., in favor of activation (Lorimer et al., 1976). The precise role of the divalent metal ion has not yet been established. There is no unequivocal evidence that the divalent metal ion coordinates with and stabilizes the enzyme-bound carbamate. The possibility that the effect of the divalent metal ion upon activation is achieved through a conformational change brought about by its binding at some other site has not yet been excluded. However, the need for CO_2 to be present for tight Mn^{2+} binding (Miziorko & Mildvan, 1974), the interacting influence of $[Mg^{2+}]$ and pH upon the equilibrium position of the activation reaction (Lorimer et al., 1976), and the fact that CABP "locks on" not only the activator CO_2 but also the divalent metal ion into positions which prevent their ready exchange with unbound ligand (Miziorko, 1979; Miziorko & Sealy, 1980) are observations most readily and simply explained by invoking a direct interaction between the carbamate and the divalent metal ion (Scheme I). The structure of the activator CO_2 peptide with the lysyl carbamate bracketed by acidic residues encourages the belief that it is the site for divalent metal ion binding. This view provides an explanation for the sensitivity of the activation reaction to changes in the pH and divalent metal ion concentration.

Evolutionary Relationships. The sequence of the activator CO_2 peptide described here is sufficiently noteworthy that one wonders to what extent it has been conserved throughout

Scheme I



evolution. The complete sequence of the large subunit of the enzyme from *Zea mais* has recently been determined (McIntosh et al., 1980). A sequence (residues 196–212) entirely homologous to that of the spinach enzyme activator CO_2 peptide is present. The lysyl residue bearing the activator CO_2 (residue 202) is 26 residues removed from the lysine (176) which Hartman et al. (1978) have identified by affinity labeling techniques as being within the active-site domain. This result imples that the activator CO_2 may not be very distant from the RuBP binding site and raises the intriguing possibility that the carbamate–divalent metal ion complex might form part of the catalytic site.

It is clearly no great evolutionary leap from spinach to maize. However, it is worth noting that the two plants are taxonomically and physiologically distinct. Spinach is dicotyledonous and relies entirely on the classic C_3 pathway of photosynthesis. Maize is monocotyledonous and uses the C_4 pathway of photosynthesis (Hatch & Slack, 1970). The conservation of the activator CO_2 peptide sequence between these two plants encourages the belief that this sequence might have been conserved over a much longer period of evolution. The ability to undergo activation by CO_2 and Mg^{2+} appears to be a property common to all RuBP carboxylases. The L_2 form of the enzyme (a dimer of two 57 000-dalton subunits) found in *Rhodospirillum rubrum* (a photosynthetic bacterium) is activated in a similar manner, although the kinetic details are a little different (Christeller & Laing, 1978). O'Leary et al. (1979) have obtained ^{13}C NMR evidence consistent with the formation of a carbamate on the enzyme from *R. rubrum*. Whether or not a sequence similar to the activator CO_2 peptide from spinach and maize can be detected in the *R. rubrum* enzyme waits to be determined.

Acknowledgments

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References

Christeller, J. T., & Laing, W. A. (1978) *Biochem. J. 173*, 467.

Ewing, S. P., Lockshon, D., & Jencks, W. P. (1980) *J. Am. Chem. Soc. 102*, 3072.

Hartman, F. C., Norton, I. L., Stringer, C. D., & Schloss, J. V. (1978) in *Photosynthetic Carbon Assimilation* (Siegelman, H. W., & Hind, G., Eds.) p 245, Plenum Press, New York.

Hatch, M. D., & Slack, C. R. (1970) *Annu. Rev. Plant Physiol. 21*, 141.

Hunkapiller, M. W., & Hood, L. E. (1978) *Biochemistry 17*, 2124.

Hunkapiller, M. W., & Hood, L. E. (1980) *Science (Washington, D.C.) 207*, 523.

Johnson, N. D., Hunkapiller, M. W., & Hood, L. E. (1979) *Anal. Biochem. 100*, 335.

Lorimer, G. H. (1979) *J. Biol. Chem. 254*, 5599.

Lorimer, G. H., & Miziorko, H. M. (1980) *Biochemistry 19*, 5321.

Lorimer, G. H., Badger, M. R., & Andrews, T. J. (1976) *Biochemistry 15*, 529.

Lorimer, G. H., Badger, M. R., & Andrews, T. J. (1977) *Anal. Biochem. 78*, 66.

McIntosh, L., Poulsen, C., & Bogorad, L. (1980) *Nature (London) 288*, 556.

Miziorko, H. M. (1979) *J. Biol. Chem. 254*, 270.

Miziorko, H. M., & Mildvan, A. S. (1974) *J. Biol. Chem. 249*, 2743.

Miziorko, H. M., & Sealy, R. C. (1980) *Biochemistry 19*, 1167.

O'Leary, M. H., Jaworski, R. J., & Hartman, F. C. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 673.

Paulsen, J. M., & Lane, M. D. (1966) *Biochemistry 5*, 2350.

Pierce, J., Tolbert, N. E., & Barker, R. (1980) *Biochemistry 19*, 934.