Greene, L. A., Shooter, E. M., & Varon, S. (1969) *Biochemistry 8*, 3735.
Hummel, B. C. W. (1959) *Can. J. Biochem. Physiol. 37*, 1393.
Jeng, I. M., Andres, R. Y., & Bradshaw, R. A. (1979) *Anal. Biochem. 92*, 482.
Morgan, P. H., Robinson, N. C., Walsh, K. A., & Neurath, H. (1972) *Proc. Natl. Acad. Sci. U.S.A. 69*, 3312.
Murphy, R. A., Saide, J. D., Blanchard, M. H., & Young, M. (1977a) *Proc. Natl. Acad. Sci. U.S.A. 74*, 2330.
Murphy, R. A., Saide, J. D., Blanchard, M. H., & Young, M. (1977b) *Proc. Natl. Acad. Sci. U.S.A. 74*, 2672.
Orenstein, N. S., Dvorak, H. A., Blanchard, M. H., & Young, M. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 5497.
Pantazis, N. J., Murphy, R. A., Saide, J. D., Blanchard, M. H., & Young, M. (1977) *Biochemistry 16*, 1525.
Pattison, S. E., & Dunn, M. F. (1975) *Biochemistry 14*, 2733.
Pattison, S. E., & Dunn, M. F. (1976a) *Biochemistry 15*, 3691.
Pattison, S. E., & Dunn, M. F. (1976b) *Biochemistry 15*, 3696.
Server, A. C., & Shooter, E. M. (1978) *Adv. Protein Chem. 31*, 339.
Shaw, E. (1967) *Methods Enzymol. 11*, 677.
Sherwood, L. M., & Potts, J. T. (1965) *J. Biol. Chem. 240*, 3799.
Smith, A. P., Greene, L. A., Fisk, H. R., Varon, S., & Shooter, E. M. (1969) *Biochemistry 8*, 4918.
Varon, S., Nomura, J., & Shooter, E. M. (1967) *Biochemistry 6*, 2202.
Young, M. (1979) *Biochemistry 18*, 3050.
Young, M., Saide, J. D., Murphy, R. A., & Blanchard, M. H. (1978) *Biochemistry 17*, 1490.

Carbamate Formation on the ϵ-Amino Group of a Lysyl Residue as the Basis for the Activation of Ribulosebisphosphate Carboxylase by CO_2 and Mg^{2+} †

George H. Lorimer* and Henry M. Miziorko*

ABSTRACT: Ribulosebisphosphate carboxylase (EC 4.1.1.39) from spinach leaves forms a stable complex of enzyme·activator CO_2·Mg·carboxyarabinitol bisphosphate. The CO_2 molecule of this complex does not readily exchange with free CO_2. The enzyme·activator $^{14}CO_2$·Mg·carboxyarabinitol bisphosphate complex was prepared free of unbound $^{14}CO_2$, and a vast molar excess of $^{12}CO_2$ was added. This mixture was added to a solution of diazomethane in methyl Cellosolve. The ^{14}C radioactivity became "fixed" to the enzyme with a yield of about 50%. The fixation of the activator CO_2 to the enzyme by diazomethane treatment occurred in a highly specific manner. The methylated enzyme·activator $^{14}CO_2$ did not undergo exchange with $^{12}CO_2$ and remained bound to enzyme denatured with sodium dodecyl sulfate. The activator $^{14}CO_2$ was bound exclusively to the large catalytic subunit. Prolonged enzymatic hydrolysis (protease from *Streptomyces griseus*) of the methylated enzyme·activator $^{14}CO_2$ followed by amino acid analysis yielded a single peak of ^{14}C radioactivity. The compound in this peak subsequently cochromatographed with genuine N^{ϵ}-(methoxycarbonyl)lysine in a variety of chromatographic systems (ion-exchange chromatography, reverse-phase, high-pressure liquid chromatography, and thin-layer chromatography with two solvent systems). Acid hydrolysis (6 N HCl, 24 h, 115 °C) of the radioactive compound resulted in the volatilization of the ^{14}C. Fixation of the activator CO_2 to the enzyme by diazomethane is due to the esterification of the N^{ϵ}-lysyl carbamate with the formation of N^{ϵ}-(methoxycarbonyl)lysine. Thus activation of ribulosebisphosphate carboxylase is achieved by the formation of a carbamate on the ϵ-amino group of a lysyl residue on the catalytic subunit.

Kinetic (Lorimer et al., 1976; Badger & Lorimer, 1976) and physical studies (Miziorko & Mildyan, 1974) have established that the activation of RuBP[1] carboxylase involves the ordered addition of CO_2[2] and Mg^{2+} (eq 1), with the addition of CO_2 representing the rate-limiting step.

$$\underset{\text{(inactive)}}{\text{enzyme} + CO_2 \rightleftharpoons \text{enzyme}\cdot CO_2 + Mg^{2+}} \rightleftharpoons \underset{\text{(active)}}{\text{enzyme}\cdot CO_2\cdot Mg} \quad (1)$$

The activator CO_2 is distinct from the CO_2 which ultimately becomes fixed during carboxylation. This point has been recently established through kinetic turnover experiments (Lorimer, 1979) and the demonstration (Miziorko, 1979) that a very stable quaternary complex of enzyme·activator CO_2·Mg·2-carboxyarabinitol 1,5-bisphosphate can be formed. CABP is an analogue of 2-carboxy-3-ketoarabinitol 1,5-bisphosphate, which is thought to be the six-carbon intermediate of the carboxylase reaction (Siegel & Lane, 1973; Pierce et al., 1980). CABP binds very tightly to the enzyme with a K_D in the order of 10^{-11} M (Pierce et al., 1980). It is reasonable

† From the Central Research and Development Department, E. I. du Pont de Nemours and Company, Experimental Station E328, Wilmington, Delaware 19898 (G.H.L.), and the Department of Biochemistry, The Medical College of Wisconsin, Milwaukee, Wisconsin 53226 (H. M.M.). *Received May 14, 1980.* This is Central Research and Development Department Contribution No. 2729. This work was in part supported by U.S. Department of Agriculture Grant 5901-0410-9-0339-0 and National Science Foundation Grant PCM-7911474. H.M.M. is a U.S. Public Health Service Research Career Development Awardee (AM 00645).

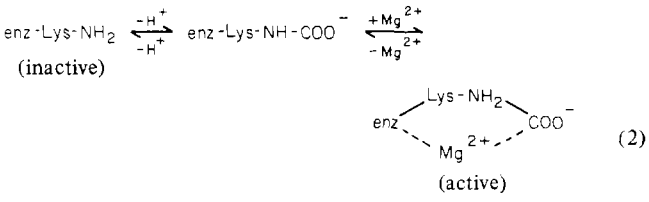
[1] Abbreviations used: RuBP, ribulose 1,5-bisphosphate; CABP, 2-carboxyarabinitol 1,5-bisphosphate; bicine, *N,N'*-bis(2-hydroxyethyl)-glycine; $NaDodSO_4$, sodium dodecyl sulfate.

[2] CO_2, rather than HCO_3^-, is considered to be the species involved in both activation (Lorimer et al., 1976) and catalysis (Cooper et al., 1969). At the pH values used in the experiments here, HCO_3^- is the predominant species in solution. The term CO_2 will be used to refer to the mixture of CO_2 and HCO_3^-.

0006-2960/80/0419-5321$01.00/0

to assume that the carboxyl group of CABP occupies the same site on the enzyme as would the substrate CO_2 molecule. Therefore, the CO_2 of the quaternary enzyme·CO_2·Mg·CABP complex must occupy another site (Miziorko, 1979).

As an explanation for the pH dependency of the activation kinetics, for the fact that CO_2 (and not HCO_3^-) is the species responsible for activation (Lorimer et al., 1976), and for the increased affinity of enzyme for metal after the CO_2 activator site is occupied (Miziorko & Mildvan, 1974), reaction of the activator CO_2 with an ϵ-amino group of a lysyl residue was proposed (eq 2) (Lorimer et al., 1976).



(2)

A recent ^{13}C NMR study with the simpler dimeric form of the enzyme from *Rhodospirillum rubrum* yielded evidence consistent with this proposal (O'Leary et al., 1979). When $^{13}CO_2$ and Mg^{2+} were added, an additional resonance appeared in the spectrum. The position of this resonance was consistent with it being due to a carbamate. However, the question as to whether or not this resonance was specifically due to carbamate formation by the activator CO_2 was not addressed.

Carbamates of the type under discussion here are much too labile to withstand the conventional techniques of protein chemistry, without prior modification. It was therefore essential to specifically modify the putative carbamate in such a manner that it could withstand the rigors of analysis. In this report we describe such a method and how we have used it to demonstrate that the activation of RuBP carboxylase by CO_2 is indeed associated with the formation of a carbamate on the ϵ-amino group of a lysyl residue on the large catalytic subunit.

Experimental Procedures

Materials

RuBP carboxylase from spinach leaves was purified, activated, and assayed as previously described (Lorimer et al., 1976, 1977). Enzyme protomer (the 70 000-dalton species containing one large subunit and one small subunit) was determined at 280 nm by using an extinction coefficient of 1.15×10^5 M^{-1} cm^{-1}, on the basis of data reported elsewhere (Paulsen & Lane, 1966). This value was found to be applicable to protomer dissociated in $NaDodSO_4$. It was assumed to be applicable to the methylated protomer dissociated in $NaDodSO_4$.

RuBP and protease from *Streptomyces griseus* were obtained from Sigma Chemical Co. Sephadex was purchased from Pharmacia Fine Chemicals. Bio-Rad Labs supplied the ion-exchange resins. *N*-Methyl-*N'*-nitro-*N*-nitrosoguanidine came from Aldrich Chemical Co. $Na_2{}^{14}CO_3$ was the product of either Amersham/Searle or New England Nuclear. A millimole size diazomethane generator was purchased from Pierce Chemical Co. N^{α}-(*tert*-butyloxycarbonyl)-L-lysine was obtained from Chemical Dynamics Corp., South Plainfield, NJ, and methyl chloroformate from Eastman Kodak Co.

Methods

Synthesis of Carboxypentitol 1,5-Bisphosphate. Carboxypentitol 1,5-bisphosphate was prepared by following a published procedure (Siegel & Lane, 19733). No attempt was made to separate the two epimers which were formed as a result of this procedure. However, CABP has been identified as the epimer which binds so tenaciously to the enzyme (Pierce et al., 1980). For simplicity, we will refer to the mixture of epimers as CABP.

Synthesis of Diazomethane.[3] About 200 mg of *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine and about 500 μL of water were placed in the inner tube of the diazomethane generator. Two milliliters of methyl Cellosolve was placed in the outer tube, and the apparatus was immersed in an ice bath. After the reactants were cooled, diazomethane was liberated upon injection of about 500 μL of ice-cold 5 N NaOH. The reaction was allowed to proceed at 0 °C for about 20 min and then the apparatus was placed in a bed of dry ice for 10 min. During this period, the yellow color intensified perceptibly as more diazomethane (bp −23 °C) became dissolved in the methyl Cellosolve. On the basis of the approximately 80% yield reported for the synthesis of diazomethane from *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (Regitz, 1977), the final concentration of diazomethane was estimated to be very roughly 0.5 M.

Formation of the Methylated Enzyme·Activator $^{14}CO_2$. A 500-μL aliquot containing about 2.5 mg of the enzyme·activator $^{14}CO_2$·Mg·CAPB complex in 50 mM bicine–NaOH and 20 mM $NaHCO_3$, pH 8.2, was chilled on ice and then added dropwise with stirring to 2 mL of approximately 0.5 M diazomethane in methyl Cellosolve. The reaction was allowed to proceed for about 30 min at 0 °C, during which time the yellow color faded and a slight turbidity developed. The mixture was then transferred to 3 mL of ice-cold H_2O, whereupon the yellow color disappeared completely and a flocculent precipitate formed. This was recovered by centrifugation and resuspended in H_2O with a vortex mixer.

For determination of the yield of methylated enzyme·activator $^{14}CO_2$, a 100-μL aliquot of the above suspension was added to 400 μL of 0.5 M Tris-HCl, pH 8.0, containing 1% (w/v) $NaDodSO_4$. The resultant solution was run through a 1.5 × 5.3 cm column of Sephadex G-25 equilibrated with 0.050 M Tris-HCl and 0.5% (w/v) $NaDodSO_4$, pH 8.0. The protein fraction was collected. Protein was measured as above and ^{14}C radioactivity determined by liquid-scintillation counting.

Isolation of the Methylated Activator CO_2 Binding Residue. Eight milligrams of the methylated enzyme·activator $^{14}CO_2$ in the form of a precipitate was suspended in 5 mL of 25 mM NH_4HCO_3, pH 7.8. One milligram of the nonspecific protease from *Streptomyces griseus* was added, followed by 0.2 mL of ethanol. The mixture was incubated at 23 °C for 92 h with continuous stirring. Under similar reaction conditions, digestion with this protease has reduced substantial amounts of denatured protein to free amino acids (Miziorko et al., 1975; Miziorko & Lane, 1977). After digestion, a small quantity of insoluble material was removed by centrifugation. The supernatant solution was lyophilized. The residue was dissolved in 1 mL of 25 mM NH_4HCO_3, pH 7.8, and chromatographed on a 1.5 × 15 cm column of Sephadex G-25 equilibrated with the same buffer. The ^{14}C-labeled material was completely included, indicating that it was now in a low-molecular-weight form (<1000 daltons). The peak of ^{14}C radioactivity, containing 70% of the ^{14}C radioactivity originally trapped as the methylated enzyme·activator $^{14}CO_2$, was pooled.

Further purification was achieved on a Beckman Model

[3] *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 described here should only be conducted in a well-ventilated hood behind a safety shield.

120B amino acid analyzer equipped for standard single-column analysis (0.9 × 30 cm column packed with Durrum DC-6A resin). The length of the first buffer cycle was doubled to improve resolution. This caused the peak of radioactivity to emerge before the buffer change (Figure 2).

Identification of the Methylated Activator CO_2 Binding Residue as N^{ϵ}-(Methoxycarbonyl)lysine. The ^{14}C-labeled material, recovered after chromatography with the amino acid analyzer, was applied to a small column of Dowex 50, eluted with NH_4OH, and dried under vacuum. This then was subjected to analyses by thin-layer chromatography and high-pressure liquid chromatography.

(*a*) *Thin-Layer Chromatography* (*Figure 3*). Samples of the ^{14}C-labeled material were applied to Eastman silica gel sheets along with 4 μg of authentic N^{ϵ}-(methoxycarbonyl)-lysine. Chromatograms were developed in (i) ethanol/NH_4OH/water (7:1:2 v/v) or (ii) butanol/acetic acid/water (4:1:1 v/v). Carrier N^{ϵ}-(methoxycarbonyl)lysine was visualized by spraying the sheet with ninhydrin. ^{14}C radioactivity was determined by cutting the sheets into 5-mm sections and counting the sections in a liquid-scintillation counter. In both solvent systems, the ^{14}C radioactivity migrated as a single peak, coincident with the ninhydrin-positive spot. Seventy-five percent of the radioactivity loaded was recovered in this position.

(*b*) *Reverse-Phase High-Pressure Liquid Chromatography* (*Figure 4*). A sample of the ^{14}C-labeled material was loaded onto an Altex Ultrasphere-ODS column (5 μm; 4.6 × 250 mm) equilibrated with 20 mM H_3PO_4 and eluted at a flow rate of 1 mL/min with a gradient ranging from 0 to 35% acetonitrile over 14 min. The effluent was collected in fractions which were counted in the usual manner. One peak of ^{14}C radioactivity, accounting for 85% of the ^{14}C loaded, eluted from the column at the same position observed for authentic N^{ϵ}-(methoxycarbonyl)lysine. The chromatographic behavior of authentic N^{ϵ}-(methoxycarbonyl)lysine was determined by using identical conditions but monitoring the absorbance of the effluent at 205 nm.

Synthesis and Characterization of N^{ϵ}-(Methoxycarbonyl)-L-lysine. This was achieved by using a modification of a previously published procedure (Chibnall & Spahr, 1958). Ten millimoles (2.46 g) of N^{α}-(*tert*-butyloxycarbonyl)-L-lysine was dissolved in 10 mL of water and the pH adjusted to 9.5 with NaOH. Methyl chloroformate was added dropwise while the pH was maintained between 7 and 10 with NaOH, until no ninhydrin-positive material could be detected. About 20 mmol of methyl chloroformate was generally required. The solution was adjusted to pH <1.0 with concentrated HCl and allowed to stand overnight at room temperature. After the solution was taken to dryness, the salty residue was twice extracted with ethanol. Petroleum ether was added to the extract in excess, and crystals were allowed to form overnight. These were filtered, washed with petroleum ether, and dried under vacuum. The yield was 55–60%. Anal. Calcd for N^{ϵ}-(methoxycarbonyl)-L-lysine hydrochloride ($C_8H_{17}N_2O_4Cl$): C, 39.92; H, 7.12; N, 11.64. Found C, 40.07; H, 6.89; N, 11.67. Thin-layer chromatography on silica gel revealed a single ninhydrin-positive spot, R_f 0.57 in ethanol/NH_3/water (7:2:1) and R_f = 0.48 in 1-propanol/water/*n*-butyl acetate/acetic acid/pyridine (120:60:20:4:1). ^{13}C NMR gave a spectrum entirely consistent with this material being N^{ϵ}-(methoxycarbonyl)lysine. The ^1H-decoupled, ^{13}C spectrum was obtained at 29 ± 1 °C, pH 1.0, with a sweep width of 6024 Hz, by using dioxane at 67.4 ppm as an internal standard. The following chemical shifts (in ppm) were assigned HOOC-[172.8]-HC[53.7](NH_3^+)-CH_2[29.2]-CH_2[22.2]-CH_2-[30.2]-CH_2[40.8]-NH-COO[160.0]-CH_3[53.2].

Results

Specific Trapping of the Enzyme·Activator $^{14}CO_2$ Complex with Diazomethane. Some years ago, Akoyunoglou et al. (1967) reported that a RuBP carboxylase-$^{14}CO_2$ complex could be stabilized by treatment with diazomethane. Upon tryptic digestion of the resultant methylated enzyme·$^{14}CO_2$, the radioactivity was shown to be associated predominantly with one peptide, implying that the reactions of $^{14}CO_2$ with the enzyme and of the enzyme·$^{14}CO_2$ complex with CH_2N_2 were both highly specific. While both reactions may have proceeded at some specific site upon the enzyme, the possibility that the $^{14}CO_2$ first dissociated from the enzyme, later to associate and to be fixed at another site, was not excluded. In the absence of any indication of yield or stoichiometry, the demonstration that only one peptide becomes radioactive merely shows that there is one site on the enzyme, which upon treatment with an ethereal solution of diazomethane is especially susceptible to reaction. The problem of nonspecific fixation of CO_2 to the enzyme by treatment with CH_2N_2 limits the utility of this approach. For example, treatment of either boiled or Na-DodSO$_4$-denatured enzyme with an ethereal solution of CH_2N_2 in the presence of $^{14}CO_2$ leads to the trapping of just as much ^{14}C as does treatment of the native enzyme (Lorimer, unpublished results). Clearly, it was imperative to develop a strategy which would circumvent the problem of nonspecificity.

The discovery that the quaternary enzyme·activator $^{14}CO_2$·Mg·CABP complex is so stable that it does not undergo exchange with a vast molar excess of $^{12}CO_2$ (Miziorko, 1979) suggested to us a simple strategy which circumvented the problem of nonspecificity in the CH_2N_2 fixation step. The enzyme·activator $^{14}CO_2$·Mg·CABP complex was prepared and isolated by gel filtration chromatography in a buffer containing a vast molar excess of $^{12}CO_2$. The presence of 1 mol of $^{14}CO_2$ per mol of protomer was confirmed at this stage (Table I), thus eliminating the possibility that the $^{14}CO_2$ was bound nonspecifically elsewhere. The mixture of enzyme·activator $^{14}CO_2$·Mg·CABP in cold $^{12}CO_2$ was then added to a solution (approximately 0.5 M) of CH_2N_2 in ethylene glycol monomethyl ether. Clearly only those molecules of activator $^{14}CO_2$ which remain bound at the activator site will be fixed by CH_2N_2. Those which dissociate become diluted by the vast excess of cold $^{12}CO_2$.

The validity of the strategy outlined above was demonstrated with the following experiment (Table I). The enzyme·activator CO_2·Mg·CABP complex was prepared by using either $^{12}CO_2$ or $^{14}CO_2$, and the complexes were isolated by gel filtration vs. buffer containing 20 mM HCO_3^-. The total radiospecific activity [total ^{14}C dpm/mol (bound CO_2 + unbound CO_2)] was determined from the sample containing the enzyme-bound $^{14}CO_2$. The sample with enzyme-bound $^{12}CO_2$ was then brought to approximately the same total radiospecific activity by the addition of the appropriate amount of carrier-free $^{14}CO_2$. At this stage, the only difference between the two samples lies in the location of the $^{14}CO_2$. In the test case (A), all of the $^{14}CO_2$ is enzyme bound, whereas in the control (B), all of the $^{14}CO_2$ is unbound or at most nonspecifically bound. If the reaction with CH_2N_2 proceeded completely nonspecifically, the specific radioactivity of the methylated enzyme·$^{14}CO_2$ product would have been the same in both cases. On the other hand, complete and specific trapping would have resulted in very different radiospecific activities, with the radiospecific activity of the test sample approaching or equaling the radiospecific activity of the enzyme·activator $^{14}CO_2$·

Table I: Specific Trapping of Enzyme·Activator $^{14}CO_2$ with Diazomethane[a]

expt	mol of $^{14}CO_2$/mol of protomer in enzyme·activator $^{14}CO_2$·Mg·CABP complex	molar excess of free (CO_2 + HCO_3^-)/ bound CO_2	specific radioactivity (^{14}C, dpm/nmol) enzyme·activator $^{14}CO_2$·Mg·CABP (bound CO_2)	total free + bound CO_2	methylated enzyme·$^{14}CO_2$	yield
1A	1.2	273 (3.9)[b]	2006	8.6	1117 (130)[c]	0.56[d]
1B	0	352 (5.1)	0	15.2	20	1.32[e]
2A	1.1	238 (3.4)	2006	11.7	1031 (88)	0.51[d]
2B	0	200 (2.9)	0	23.7	53	2.20[e]
3A	1.2	656 (9.4)	2251	4.2	849 (202)	0.38[d]
3B	0	604 (8.7)	0	5.6	17.8	3.20[e]
4A[f]	0.98	4241 (9.9)	3480	0.8	1860 (2325)	0.53[d]
4B[f]	0	3903 (9.1)	0	19.3	144	7.49[e]

[a] RuBP carboxylase was first incubated for 15 min at 25 °C in 50 mM bicine–NaOH, pH 8.2, 20 mM $MgCl_2$, and either (A) 10 mM $NaH^{14}CO_3$ of defined specific radioactivity or (B) 10 mM $NaH^{12}CO_3$. A small aliquot was then removed for determination of carboxylase activity. To the remainder was then added an approximately 10-fold molar excess (with respect to enzyme protomer) of CABP and the mixture incubated for a further 60 min. The carboxylase activity, determined after incubation with CABP, was never greater than 2% of the initial activity, indicating that at least 98% of the protomer molecules were occupied by CABP. The resultant enzyme·activator CO_2·Mg·CABP was then subjected to gel filtration on a column of Sephadex G-25 equilibrated with 50 mM bicine–NaOH and 20 mM $NaHCO_3$, pH 8.2. (In the case of large-scale preparations involving micromolar quantities of enzyme protomer, the enzyme·activator CO_2·Mg·CABP complex was first precipitated with 1.2 volumes of saturated $(NH_4)_2SO_4$. The precipitate was recovered by centrifugation, dissolved in a small volume of buffer, and subjected to gel filtration as above.) The protein concentration was then adjusted to 4–5 mg/mL by the addition of 50 mM bicine–NaOH and 20 mM $NaHCO_3$, pH 8.2. At this stage the following were determined: column 2, mol of $^{14}CO_2$/mol of protomer in enzyme·activator CO_2·Mg·CABP complex; column 3, molar excess of free (CO_2 + HCO_3^-) over enzyme-bound activator CO_2; column 5, the total radiospecific activity (free CO_2 + HCO_3^- + enzyme-bound activator CO_2). On the basis of this last determination, sufficient carrier-free $^{14}CO_2$ was added to (B), i.e., to the enzyme·activator $^{12}CO_2$·Mg·CABP complex, to give a comparable total radiospecific activity. The enzyme·activator $^{14}CO_2$·Mg·CABP complex was methylated with diazomethane and the yield of methylated enzyme·activator $^{14}CO_2$ determined as described under Methods. [b] The values in parentheses refer to the molar ratio of unbound real CO_2 to bound activator CO_2. [c] The values in parentheses refer to the ratio of specific radioactivity of the methylated enzyme·$^{14}CO_2$ to that of the total CO_2 (bound + unbound). [d] The yield is expressed as the mol of specifically trapped activator $^{14}CO_2$ per mol of methylated protomer and is based upon the specific radioactivity of the $^{14}CO_2$ in the enzyme·activator $^{14}CO_2$·Mg·CABP complex. [e] In the case of the control experiments (B), the yield is expressed as the mol of nonspecifically trapped $^{14}CO_2$ per mol of methylated protomer and is based upon the specific radioactivity of the total CO_2 (bound + unbound). [f] In this experiment, the enzyme·activator CO_2·Mg·CABP complex was isolated in a buffer of 0.2 M $NaHCO_3$/Na_2CO_3, pH 9.00.

Mg·CABP complex. The results of such an experiment are given in Table I. Several points deserve comment.

We have tried to reproduce the fixation procedure with diethyl ether as solvent for the CH_2N_2 in place of methyl Cellosolve without success. When the enzyme was introduced into the mixture of CH_2N_2 in diethyl ether, it immediately precipitated. This was not the case with methyl Cellosolve. Provided the pH of the solution containing the enzyme·activator $^{14}CO_2$·Mg·CABP complex was greater than about 8.0, the protein remained in solution with only very slight turbidity developing toward the end of the reaction period. It will be noted that the fixation reaction occurs in a solution containing upwards of 80% (v/v) of methyl Cellosolve. It is improbable that the enzyme maintains its native structure in such a solution. However, the specific reaction of the activator CO_2 with CH_2N_2 is clearly favored by conditions which keep the enzyme in solution. For this, a rather high concentration of methyl Cellosolve was necessary. When, following the reaction of the enzyme with CH_2N_2, the concentration of methyl Cellosolve was lowered to about 36% (v/v) by dilution with water, the protein precipitated. Thus the conditions for the specific fixation of the enzyme·activator $^{14}CO_2$ in high yield appear to be quite critical.

The ^{14}C radiospecific activity of the methylated enzyme·activator $^{14}CO_2$ is from 85 to 2300 times the radiospecific activity of the total CO_2 originally present, indicating that the enzyme·activator $^{14}CO_2$ did not come into equilibrium with the free CO_2 before reacting with CH_2N_2. On the contrary, the specific radioactivity of the methylated enzyme·activator $^{14}CO_2$ approaches that of the $^{14}CO_2$ in the original quaternary complex of enzyme·activator $^{14}CO_2$·Mg·CABP. The results indicate that about half of the activator CO_2 molecules are "fixed" by the diazomethane treatment.

The results of the control experiments (B in Table I) underline the necessity for circumventing the problem of nonspecificity. Starting with a solution of enzyme·activator $^{12}CO_2$·Mg·CABP in $^{14}CO_2$ at pH 8.2, 1–2 mol of nonspecific CO_2 per mol of protomer was trapped with diazomethane. While some of these CO_2 molecules might have become fixed at the activator site, there is certainly no guarantee that this was exclusively so. The results of these controls also eliminate the possibility that dissociation of the enzyme·activator $^{14}CO_2$·Mg·CABP complex was followed by multiple nonspecific reassociation and reaction with CH_2N_2.

The values reported in Table I for the molar excess of unbound CO_2 over bound CO_2 are based upon the total inorganic carbon, i.e., CO_2 plus HCO_3^-. It was assumed, if the activator $^{14}CO_2$ dissociated from the enzyme, that it would come into equilibrium with the total $^{12}CO_2$ (CO_2 + HCO_3^-) of the medium. However, this equilibrium is established rather slowly, and it was conceivable that the dissociated $^{14}CO_2$ only mixed with the real $^{12}CO_2$ which at pH 8.2 was only present in threefold to fourfold molar excess. In experiment A of Table I, the molar ratio of free real $^{12}CO_2$ to bound $^{14}CO_2$ was increased to 10 and that of free total $^{12}CO_2$ (CO_2 + HCO_3^-) to bound $^{14}CO_2$ to 4000. The specific radioactivity of the methylated enzyme $^{14}CO_2$ was 2000 times that of the total $^{14}CO_2$, assuming complete equilibration of all species or 5.5 times that of the real CO_2, assuming no equilibration between CO_2 and HCO_3^-. A further point was brought out by experiment 4B. By increases in pH and the total HCO_3^- concentration, the extent of nonspecific fixation can be considerably enhanced.

From the results of the experiments described in Table I, it can be concluded that the enzyme $^{14}CO_2$ was fixed by the CH_2N_2 treatment in a highly specific manner at the same site to which the $^{14}CO_2$ was originally bound, i.e., at the CO_2 activator site.

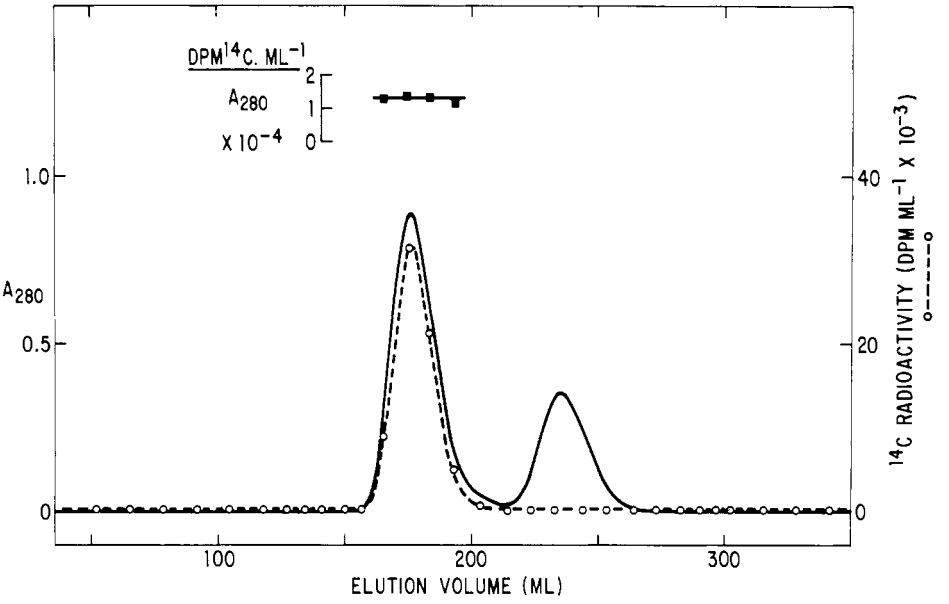


FIGURE 1: Isolation of the methylated large subunit activator $^{14}CO_2$ complex by NaDodSO$_4$–gel filtration chromatography. The solid line (—) represents the continuous A_{280} trace from the UV detector monitoring the column effluent; (○) ^{14}C radioactivity; (■) ^{14}C radiospecific activity of the methylated large subunit activator $^{14}CO_2$ complex expressed as dpm of ^{14}C mL^{-1} per A_{280} unit. RuBP carboxylase (77 mg or 1.4 μmol of protomer) was incubated for 15 min at 25 °C with 665 μmol of NaOH–*N*,*N*′-bis(2-hydroxyethyl)glycine (bicine), pH 8.21, 250 μmol of $MgCl_2$, and 96.5 μmol of $^{14}CO_2$ (2006 dpm/nmol), in a total volume of 14 mL. Carboxylase activity determined after 13 min was 1.02 μmol·min^{-1} (mg of protein)$^{-1}$ (100%). Then 10 μmol of CABP was added and the incubation continued for a further hour at 25 °C. Carboxylase activity was then 0.015 μmol min^{-1} (mg of protein)$^{-1}$ (about 1.5%). The sample was cooled on ice and the enzyme·activator $^{14}CO_2$·Mg·CABP complex precipitated by the addition of 1.22 volumes of saturated $(NH_4)_2SO_4$. The precipitate was recovered by centrifugation and dissolved in about 3 mL of 50 mM bicine–NaOH and 5 mM dithiothreitol, pH 8.2. The protein solution was passed through a 2.6 × 9.5 cm column of Bio-Gel P-4 equilibrated with 45 mM bicine–NaOH and 20 mM $NaHCO_3$, pH 8.2. The enzyme·activator $^{14}CO_2$·Mg·CABP complex recovered at this stage had the following properties: protein concentration = 5.4 mg mL^{-1} (77 μM protomer), mol of activator $^{14}CO_2$/mol of protomer = 1.22, mol of free $^{12}CO_2$/mol of protomer-bound $^{14}CO_2$ = 213. Twenty-six 2-mL batches of diazomethane in methyl Cellosolve were prepared as described under Methods. To each batch was added dropwise with stirring 500 μL of the above enzyme·activator $^{14}CO_2$·Mg·CABP complex containing 2.7 mg of protein, previously chilled on ice. After a further 30 min on ice, 3 mL of ice-cold H_2O was added and the precipitate which developed recovered by centrifugation. The precipitate containing the methylated enzyme·activator $^{14}CO_2$ complex was dissolved with 5 mL of 50 mM Tris-HCl, 10% (w/v) NaDodSO$_4$, and 10 mM dithiothreitol, pH 8.0, and the resultant solution run through a 2.6 × 9.5 cm column of Bio-Gel P-4 equilibrated with 50 mM Tris-HCl and 1% (w/v) NaDodSO$_4$, pH 8.0. The methylated enzyme·activator $^{14}CO_2$ complex recovered at this stage had the following properties: mol of activator $^{14}CO_2$/mol of methylated protomer = 0.48, specific ^{14}C radioactivity = 8710 dpm mL^{-1} per A_{280} unit. To 12 mL of the above methylated enzyme·activator $^{14}CO_2$ complex was added 10 μL of 2-mercaptoethanol, and the solution was incubated for about 15 min at 25 °C. Then 2 mL of 1 M iodoacetate in 1.2 M $NaHCO_3$ was added and the mixture incubated in the dark for a further 20 min. Thereafter, 150 μL of 2-mercaptoethanol was added to quench the unreacted iodoacetate and the sample applied to a 2.6 × 90 cm column of Sephadex G-100 equilibrated with 50 mM Tris-HCl and 0.5% (w/v) NaDodSO$_4$, pH 8.0. Flow rate = 20 mL/h, fraction size = 10 mL. The methylated large subunit·activator $^{14}CO_2$ complex, containing 91% of the total ^{14}C applied and more than 99% of the total ^{14}C recovered, had a constant specific ^{14}C radioactivity of 13720 dpm mL^{-1} per A_{280} unit.

The Activator CO_2 Is Bound to the Large Subunit. Stabilization of the enzyme·activator CO_2 complex in the highly specific manner described above enabled us to determine to which of the two types of subunit, large or small, the activator CO_2 was bound. After carboxymethylation, the methylated enzyme·activator $^{14}CO_2$ complex was subjected to NaDodSO$_4$–gel filtration chromatography with the result shown in Figure 1. The large and small subunits were clearly separated. NaDodSO$_4$–gel electrophoresis (data not shown) confirmed the identity of the two peaks. Of the ^{14}C radioactivity applied to the column, more than 92% was recovered. This ^{14}C radioactivity was confined exclusively (>99% of the total recovered) to the first peak which was eluted with constant radiospecific activity (constant dpm mL^{-1} per A_{280} unit). Therefore, the activator CO_2 is bound to the large subunit. In addition, the absence of ^{14}C bound to the small subunit confirms the conclusion already reached, i.e., that the fixation of the enzyme·activator $^{14}CO_2$ complex with CH_2N_2 has taken place in a highly specific manner.

The Activator CO_2 Is Bound as a Carbamate to the ε-Amino Group of a Lysine Residue. If the activation of the enzyme with CO_2 involves the formation of a carbamate on the ε-amino group of a lysyl residue (Lorimer et al., 1976), then the ability of diazomethane to "fix" the activator CO_2 to the enzyme can be attributed to the esterification of the lysyl carbamate (eq 3). The product of this esterification is N^{ϵ}-(methoxycarbonyl)lysine (Katchalski et al., 1951). To confirm this point, the methylated enzyme·activator $^{14}CO_2$ was subjected to prolonged enzymatic hydrolysis with the nonspecific protease from *Streptomyces griseus*. The chromatographic properties of the ^{14}C-labeled hydrolysis product were then compared with those of authentic N^{ϵ}-(methoxycarbonyl)lysine.

$$\sim\text{Lys-NH}_2 + CO_2 \rightleftharpoons \sim\text{Lys-NH-CO}_2 \xrightarrow{CH_2N_2} \sim\text{Lys-NH-COOCH}_3 \quad (3)$$

(*a*) *Ion-Exchange Chromatography.* Chromatography of the hydrolysate on a standard amino acid analyzer yielded a single peak of ^{14}C radioactivity (Figure 2). Typically, at least 75% of the ^{14}C-labeled material applied to the column was recovered in this single peak. Authentic N^{ϵ}-(methoxycarbonyl)lysine exhibited the same chromatographic properties (Figure 2). This procedure was used to purify the radioactive material from the hydrolysate of the methylated enzyme·activator $^{14}CO_2$. The material so obtained was subjected to further chromatographic tests.

(*b*) *Thin-Layer Chromatography.* Samples of the radioactive peak from the amino acid analyzer were subjected to

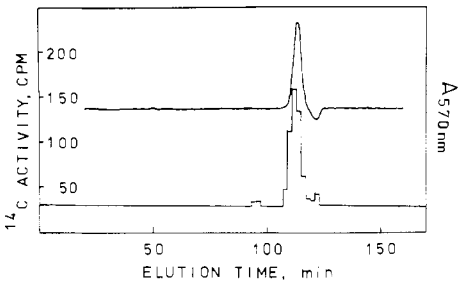


FIGURE 2: The cochromatography of the methylated activator $^{14}CO_2$ binding residue and authentic N^{ϵ}-(methoxycarbonyl)lysine. Experimental details can be found under Methods. Ion-exchanged chromatography of authentic N^{ϵ}-(methoxycarbonyl)lysine (top tracing) and of the methylated activator $^{14}CO_2$ binding residue following prolonged enzymatic hydrolysis of the methylated enzyme·activator $^{14}CO_2$ (bottom histogram).

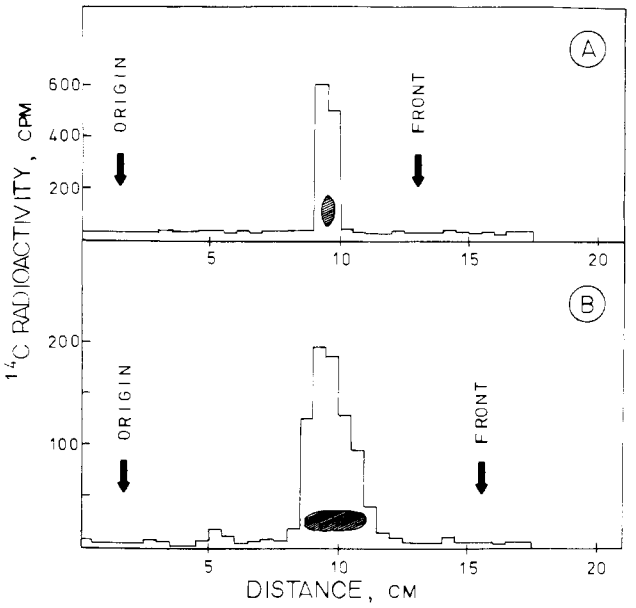


FIGURE 3: (A) Thin-layer chromatography of authentic N^{ϵ}-(methoxycarbonyl)lysine (detected by ninhydrin) and the methylated activator $^{14}CO_2$ binding residue on silica gel with ethanol/NH_4OH/H_2O (7:1:2 v/v). (B) As for (A) but with butanol/acetic acid/H_2O (4:1:1 v/v).

thin-layer chromatography along with authentic N^{ϵ}-(methoxycarbonyl)lysine. Two solvent systems were used. In both cases the ^{14}C radioactivity co-migrated with the authentic compound (Figure 3).

(*c*) *Reverse-Phase High-Pressure Liquid Chromatography.* A sample of the radioactive peak from the amino acid analyzer was subjected to reverse-phase high-pressure liquid chromatography. A single peak of ^{14}C radioactivity emerged (Figure 4). The elution profile of authentic N^{ϵ}-(methoxycarbonyl)-lysine, monitored at 205 nm and run separately under otherwise identical conditions, is shown in Figure 4. The radioactive material and the authentic compound chromatograph identically.

When the methylated activator $^{14}CO_2$ binding residue was subjected to acid hydrolysis (6 N HCl, 24 h at 115 °C), the ^{14}C radioactivity in the sample was completely volatilized (data not shown). The same treatment of authentic N^{ϵ}-(methoxycarbonyl)lysine brought about its quantitative conversion to lysine.

The results outlined in Figures 2, 3, and 4 together with the acid lability of the methylated activator $^{14}CO_2$ binding residue leave little doubt that it is indeed N^{ϵ}-(methoxycarbonyl)lysine. The activator CO_2 is therefore bound to the enzyme as a

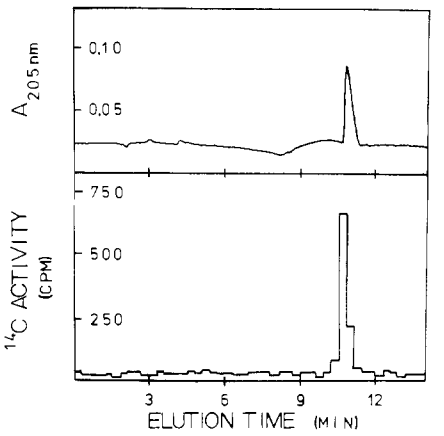


FIGURE 4: Reverse-phase high-pressure liquid chromatography of authentic N^{ϵ}-(methoxycarbonyl)lysine (top tracing) and of the methylated activator $^{14}CO_2$ binding residue, prepurified by ion-exchange chromatography (bottom histogram).

carbamate of a lysyl residue which upon esterification is converted to the ϵ-methoxycarbonyl derivative.

Discussion

The reaction of CO_2 with uncharged amines to form carbamates has been known for almost 70 years (Siegfried, 1905; Faurholt, 1924, 1925). However, with the notable exception of hemoglobin, this reaction has attracted little attention, despite its potential significance for biological systems. The generally unfavorable nature of the equilibrium has created the impression that carbamate formation, if it occurs at all in vivo, is unlikely to be of much biological importance (Edsall & Wyman, 1958). Yet, the importance of carbamate formation at the terminal α-amino groups of hemoglobin has been documented with considerable detail [for reviews see Kilmartin & Rossi-Bernardi (1973); Morrow & Gurd (1975)].

The active species in carbamate formation are known to be CO_2 and RNH_2, rather than HCO_3^- or CO_3^{2-} and RNH_3^+ (Faurholt, 1925; Caplow, 1968). Kinetic analyses of the activation of RuBP carboxylase showed that it too involved CO_2 rather than HCO_3^- (Lorimer et al., 1976). This is an important and necessary criterion to satisfy if the case for carbamate formation is to be proven. The kinetic analyses also indicated that the activator CO_2 reacted with a group, the pK of which was distinctly alkaline and whose dissociation promoted the activation reaction (Lorimer et al., 1976). Here we have demonstrated that the activator CO_2 is bound in the form of a carbamate to the ϵ-amino group of a lysine residue, thus providing a molecular explanation for the pH dependency of the activation reaction.

The carbamates formed as a result of the reaction of CO_2 with both α- and ϵ-amino groups are rather labile. There is currently no general procedure for selectively converting protein-bound carbamates to a form capable of withstanding the rigors of protein chemistry. An indication that esterification with diazomethane might prove useful in this respect was provided by Katchalski et al. (1951). They showed that the carbamates of a number of diamines could be converted to the corresponding methoxycarbonyl derivatives by treatment with diazomethane. Akoyunoglou et al. (1967) reported that diazomethane stabilized a complex formed between RuBP carboxylase and $^{14}CO_2$. However, these authors argued that carbamate formation would not be sufficiently specific to account for their results. In retrospect, it now seems highly probable that the $^{14}CO_2$ was bound to the enzyme in the form of a carbamate and that the diazomethane stabilized the ^{14}C

in the form of a carbamate ester. The procedure of Akoyunoglou et al. (1967) did not exclude the possibility that the $^{14}CO_2$ underwent migration from its original site upon the enzyme to some other site where it was fixed by esterification. In short, the important element of specificity was missing. That we have been able to circumvent this problem is entirely due to the nonexchangeability of the activator CO_2 in the enzyme·activator CO_2·Mg·CABP complex with free CO_2 in the medium, at least in the time scale under consideration. It was therefore possible to prepare this complex with $^{14}CO_2$ in such a manner that all of the ^{14}C radioactivity was bound at the activator site. By including in the reaction solution a vast molar excess of unlabeled CO_2, which would dilute out any dissociating $^{14}CO_2$, it was possible to ensure that the "fixed" ^{14}C radioactivity had not undergone migration during the harsh conditions of esterification.

Spinach RuBP carboxylase contains eight large (58 000 dalton) subunits and eight small (12 000 dalton) subunits (Rutner & Lane, 1970). Evidence has been presented that the catalytic site is located on the large subunit (Nishimura & Akazawa, 1974). A number of authors have speculated that the small subunit acts as a regulatory subunit. While some as yet undefined regulatory function may still be associated with the small subunit, it is quite clear from the results reported in Figure 1 that the activator CO_2 is bound to the large subunit. The function of the small subunit therefore remains to be determined.

The demonstration that the activator CO_2 is bound to the large (catalytic) subunit was anticipated from previous studies with the enzyme from *Rhodospirillum rubrum*. The enzyme from *R. rubrum* consists of a dimer of two large subunits and lacks the small subunit altogether (Tabita & McFadden, 1974). However, the *R. rubrum* enzyme is activated in essentially the same manner as the enzyme from spinach leaves (Christeller & Laing, 1978). Carbon-13 NMR studies of the *R. rubrum* enzyme have provided some evidence for the formation of a carbamate under the conditions known to be necessary for activation (O'Leary et al., 1979). We do not dispute that the ^{13}C NMR spectrum is typical of what one might expect for a carbamate. However, the possibility that the observed NMR signal was due to nonspecific carbamate formation was not excluded. The arguments based on the requirement for Mg^{2+} and alkaline pH's are suggestive of a relationship between the activation state of the enzyme and the ability to detect a ^{13}C NMR carbamate signal. However, these criteria apply with equal force to nonspecific carbamate formation. For example, carbamate formation in hemoglobin occurs specifically at the terminal α-amino groups at pH 7 (Matthew et al., 1977). But at pH 8.5 significant nonspecific carbamate formation on the ϵ-amino groups of lysine can be detected (Matthew et al., 1977). Thus, some caution is required in interpreting such spectra.

Our results do not directly relate to the role of the metal ion in activation and catalysis. However, they do provide an explanation for the dependency of Mn^{2+} binding upon the presence of CO_2 (Miziorko & Mildvan, 1974). In effect, carbamate formation converts a neutral or potentially cationic site on the enzyme to an anionic site to which metal ion may then bind (eq 4). The binding of the metal ion would in turn

$$E\text{-}NH_3^+ \overset{H^+}{\rightleftharpoons} E\text{-}NH_2 + CO_2 \overset{H^+}{\rightleftharpoons} E\text{-}NH\text{-}COO^- \overset{M^{2+}}{\rightleftharpoons} E\text{-}NHCOO^- \cdots M^{2+} \quad (4)$$

increase the stability of the carbamate, and there is both kinetic (Lorimer et al., 1976; Lorimer, 1979) and spectroscopic evidence (O'Leary et al., 1979; Miziorko & Sealy, 1980) which is consistent with this view.

There is evidence [discussed in Miziorko & Sealy (1980)] which points to a role for the metal ion in catalysis. If the same metal ion is involved in both activation and catalysis, it follows that the carbamate must constitute part of the catalytic site. Dissociation of this carbamate would then account for the inactivation of the enzyme. Experiments are currently in progress to prove or disprove this proposition.

In conclusion, ribulosebisphosphate carboxylase represents the first enzyme known to be activated by carbamate formation. We can only suspect that carbamate formation (and with it the modification of biological activity) may be considerably more widespread than hitherto realized.

Acknowledgments

We thank Bruce Schweiger and Doloris Hinkleman for their able technical assistance and Dr. David Glick for access to his amino acid analyzer.

References

Akoyunoglou, G., Argyroundi-Akoyunoglou, J. H., & Metheniton, H. (1967) *Biochim. Biophys. Acta 132*, 481.
Badger, M. R., & Lorimer, G. H. (1976) *Arch. Biochem. Biophys. 175*, 723.
Caplow, M. (1968) *J. Am. Chem. Soc. 90*, 6795.
Chibnall, A. C., & Spahr, P. F. (1958) *Biochem. J. 68*, 135.
Christeller, J. T., & Laing, W. A. (1978) *Biochem. J. 173*, 467.
Cooper, T. G., Filmer, D., Wishnick, M., & Lane, M. D. (1969) *J. Biol. Chem. 244*, 1081.
Edsall, J. T., & Wyman, J. (1958) *Biophysical Chemistry*, Vol. 1, pp 485–496, Academic Press, New York.
Faurholt, C. (1924) *J. Chim. Phys. Phys.-Chim. Biol. 21*, 400.
Faurholt, C. (1925) *J. Chim. Phys. Phys.-Chim. Biol. 22*, 1.
Katchalski, E., Klibanski, C. B., & Berger, A. (1951) *J. Am. Chem. Soc. 73*, 1829.
Kilmartin, J. V., & Rossi-Bernardi, L. (1973) *Physiol. Rev. 53*, 836.
Lorimer, G. H. (1979) *J. Biol. Chem. 254*, 5599.
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.
Matthew, J. B., Morrow, J. S., Wittebort, R. J., & Gurd, F. R. N. (1977) *J. Biol. Chem. 252*, 2234.
Miziorko, H. M. (1979) *J. Biol. Chem. 254*, 270.
Miziorko, H. M., & Mildvan, A. S. (1974) *J. Biol. Chem. 249*, 2743.
Miziorko, H. M., & Lane, M. D. (1977) *J. Biol. Chem. 252*, 1414.
Miziorko, H. M., & Sealy, R. C. (1980) *Biochemistry 19*, 1167.
Miziorko, H. M., Clinkenbeard, K. D., Reed, W. D., & Lane, M. D. (1975) *J. Biol. Chem. 250*, 5768.
Morrow, J. S., & Gurd, F. R. N. (1975) *CRC Crit. Rev. Biochem. 3*, 220.
Nishimura, M., & Akazawa, T. (1974) *Biochem. Biophys. Res. Commun. 59*, 584.
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.
Regitz, M. (1977) *Diazoalkane: Eigenschaften und Syn-*

thesen, p 155, Georg Thieme Verlag, Stuttgart.
Rutner, A. C., & Lane, M. D. (1970) *Biochem. Biophys. Res. Commun. 28*, 531.
Siegel, M. I., & Lane, M. D. (1973) *J. Biol. Chem. 248*, 5486.
Siegfried, M. (1905) *Hoppe-Seyler's Z. Physiol. Chem. 44*, 85.
Tabita, F. R., & McFadden, B. A. (1974) *J. Biol. Chem. 249*, 3453.

Kinetics and Mechanism of Inhibition of *Escherichia coli* Alkaline Phosphatase by Permanganate Ion†

R. A. Thomas and Jack F. Kirsch*

ABSTRACT: The interaction between *Escherichia coli* alkaline phosphatase (EC 3.1.3.1) and permanganate ion has been investigated. The kinetics of the inactivation of this enzyme by permanganate ion at pH 9.0 and 25 °C are described by a two-step mechanism in which the rapid reversible formation of a noncovalent enzyme–permanganate complex with a dissociation constant of 87 μM is followed by a first-order transformation of the complex to a second form with a first-order rate constant of 1.5 min^{-1}. The dissociation rate constant from the final inactive complex is 0.007 min^{-1}, giving an overall dissociation constant of the inactive enzyme–permanganate complex of (0.007/1.5)87 μM = 0.4 μM. The inactivation is inhibited by inorganic phosphate ion, suggesting that the association with permanganate ion most likely occurs at the active site. The effect of phosphate concentration on the inhibition of permanganate inactivation suggests a mechanism where permanganate can inactivate both the free enzyme and enzyme which has only one of the two active sites occupied by phosphate with the latter process occurring at ~40% of the rate of the former. The tetrameric enzyme, formed by dimer association at pH >7 and Zn(II) concentration >10 μM, was inactivated by permanganate with the same rate constant as the dimer. Inactivation by permanganate was significantly more rapid in preparations with low Mg(II) content. Permanganate ion does not act as an oxidant in this process since total enzyme activity is recoverable by simple dilution or by extensive dialysis of the inactivated enzyme. Thiols enhance the rate of reactivation of the permanganate-inactivated enzyme. The effect of thiol concentration on the reactivation rates suggests a mechanism where thiol rapidly reduces enzyme-bound permanganate, thus bypassing the slower dissociation step of permanganate from the inactive permanganate–enzyme complex. The slowness of the overall reaction and the two-step nature of the process suggest the possibility of a reversible covalent bond formed between permanganate ion and some residue at the active site of the enzyme—such as a manganate ester or pentacovalent adduct with the active-site serine residue.

Escherichia coli alkaline phosphatase (EC 3.1.3.1) is a zinc metalloenzyme which catalyzes the hydrolysis of phosphate monoesters. Inorganic phosphate, the product of the hydrolysis, is both a potent inhibitor and, as evidenced by ^{18}O studies (Schwartz, 1963; Applebury et al., 1970), a pseudosubstrate. Permanganate ion was examined as a possible chromophoric analogue of phosphate for transient kinetic studies with this enzyme, since Benisek (1971) had pointed out that MnO_4^- acted as a structural analogue of phosphate with aspartate transcarbamylase. Ohlsson & Wilson (1974) showed that $KMnO_4$ slowly and apparently irreversibly inactivates alkaline phosphatase in a reaction which is suppressed by phosphate, suggesting the possibility that MnO_4^- reacts at the active site. They also noted that this reaction could be reversed by the addition of thiols but were unable to distinguish between a mechanism of inactivation by oxidation vs. a stable complex formation. We report here a detailed study of the kinetics and equilibria of the interaction of $KMnO_4$ with alkaline phosphatase, including quantitative analyses of the phosphate protection and the thiol-mediated reactivation mechanism.

Materials and Methods

Enzyme Purification and Assay. Alkaline phosphatase was prepared from *E. coli* strain C90F1 by using the method of Schlesinger & Olsen (1970). The purified enzyme was stored frozen at a concentration of 5 mg/mL. The specific activity and response to MnO_4^- were unaffected by freezing. Before use, the thawed enzyme was dialyzed against 0.1 M $NaHCO_3$–Na_2CO_3, 1 mM $MgCl_2$, and 0.1 μM $ZnCl_2$ at pH 9.0 and 20 °C. The purified enzyme had a specific activity of 30 μmol of product released per min per mg of protein when assayed with 1 mM *p*-nitrophenyl phosphate in 1 M NaCl and 0.1 M Tris-HCl, pH 8.0 at 25 °C (Halford, 1972). The molar absorbancy of *p*-nitrophenolate was taken to be 16 700 at this pH (Halford, 1971). Product release was followed by using a Unicam SP 800A spectrophotometer equipped with a Texas Instruments recorder.

Protein concentration was determined at 280 nm by using $E_{1cm}^{0.1\%} = 0.72$ as determined by Malamy & Horecker (1964) for the crystalline enzyme. A molecular weight of 86 000 was used for molar concentrations (Applebury & Coleman, 1969).

Reagents. $KMnO_4$ was obtained from Matheson Coleman and Bell, mercaptoethanol was from Aldrich, and *p*-nitrophenyl phosphate (Sigma 104 phosphatase substrate) and Tris base (Trizma) were purchased from Sigma Chemical Co. All other chemicals were reagent grade products of Mallinckrodt.

All $KMnO_4$ studies were done in 0.1 M $NaHCO_3$–Na_2CO_3, 1 mM $MgCl_2$, and 0.1 μM $ZnCl_2$ at pH 9.0, hereafter referred to as 0.1 M bicarbonate pH 9 buffer. Water used for the buffer was distilled from alkaline permanganate, and buffer solutions were filtered through fine-porosity sintered glass before use. It was found that the organic amine buffers commonly used at pH 9 would reduce MnO_4^-.

† From the Department of Biochemistry, University of California, Berkeley, California 94720. *Received January 18, 1980; revised manuscript received June 30, 1980.* This investigation was supported by a grant from the National Science Foundation.

0006-2960/80/0419-5328$01.00/0