For:

Brookhaven Symposium in Biology
Number 30

CONF-780592--1

Attempts to Apply Affinity Labeling Techniques to Ribulosebisphosphate Carboxylase/Oxygenase*

Fred C. Hartman, I. Lucile Norton, Claude D. Stringer, and John V. Schloss†

Biology Division, Oak Ridge National Laboratory, and
University of Tennessee-Oak Ridge Graduate School of
Biomedical Sciences, Oak Ridge, Tennessee 37830

---

*This research was sponsored by the Division of Biological and Environmental Research, U.S. Department of Energy, under contract W-7405-eng-26 with the Union Carbide Corporation.

†Predoctoral Fellow supported by Grant GM 1974 from the National Institute of General Medical Sciences, National Institutes of Health.

DISCLAIMER

Running Title: Affinity Labeling of Ribulosebisphosphate Carboxylase/Oxygenase

Attempts to Apply Affinity Labeling Techniques to Ribulosebisphosphate Carboxylase/Oxygenase

Fred C. Hartman, I. Lucile Norton, Claude D. Stringer, and John V. Schloss

Biology Division, Oak Ridge National Laboratory, and University of Tennessee-Oak Ridge Graduate School of Biomedical Sciences, Oak Ridge, Tennessee 37830

INTRODUCTION

Although subject to uncertainties in interpretation, chemical modification is a proven method for defining structural-functional relationships in enzymes. Innumerable examples are available in which tentative conclusions based on chemical modification studies, concerning identities of active site residues and in some cases even their precise catalytic function, have been substantiated by X-ray crystallography.

The application of general (group-specific) protein reagents to Rbl-P_2* carboxylase/oxygenase has not provided detailed information about the active site, probably as a consequence of the enzyme's molecular complexity. A number of sulfhydryl reagents inactivate the enzyme, and substrate protection is observed. However, the data do not distinguish between the potential role of certain sulfhydryl groups in maintenance of tertiary and quarternary structure and their potential role in catalysis. Akazawa's group[1,2] has thoroughly studied the modification of the carboxylase/oxygenase with p-chloromercuribenzoate. At slightly alkaline pH, disruption of quarternary structure results, so there is no doubt that modification of sulfhydryls can lead to conformational changes.

---

*Abbreviations: Rbl-P_2, D-ribulose 1,5-bisphosphate; Br-butanone-P_2, 3-bromo-1,4-dihydroxy-2-butanone 1,4-bisphosphate; BrAcNHEtOP, N-bromoacetylethanolamine phosphate; Bicine, N, N^1-bis(2-hydroxyethyl)glycine.

The shortcoming of studies concerning the reaction of the enzyme with sulfhydryl reagents is that direct correlations between inactivation and modification of specific residues were not (or could not be) demonstrated because of the rather large number of sulfhydryls being modified. For example, even though Rbl-P_2 protects against inactivation by [^{14}C]iodoacetic acid, a comparative analysis of tryptic peptides derived from both inactivated and substrate-protected enzyme revealed that extensive, random modification of sulfhydryls had occurred and clear-cut differences in the labeling patterns between the two samples were not apparent[3].

More recently, the lysyl reagent pyridoxal phosphate and two arginyl reagents, 2,3-butanedione and phenylglyoxal, have been used to determine whether the carboxylase/oxygenase contains such essential residues. The encouraging results[4-6] obtained with pyridoxal phosphate will be considered later, since this reagent may be an affinity label for a binding site (probably the active site) that accommodates phosphate esters. The results[7] provided by the reaction of butanedione with carboxylase/oxygenase from barley and Pseudomonas oxalaticus were interpreted as consistent with the presence of active-site arginyl residues. Our data[8] from modification of spinach and Rhodospirillum rubrum enzymes are best interpreted in terms of 2-3 arginyl residues/protomer being essential to the stabilization of native conformation. The data from the two laboratories are not necessarily contradictory, since enzymes from different species were used and since equivalent arginyl residues may not be accessible to both reagents.

Because of the inherent disadvantages of general protein reagents due to their lack of specificity, we have attempted to systematically develop affinity labels for use in the characterization of the active site of Rbl-P_2

carboxylase/oxygenase. The major advantages of affinity labels in comparison to general protein reagents are their potentially absolute specificity for the catalytic site of a given enzyme and the mechanistic information that they can provide (volume 47 of Methods in Enzymology edited by W. B. Jakoby and M. Wilchek is an excellent reference source to successes of affinity labeling). The selectivity of an affinity-labeling reagent is a consequence of its structural similarity to substrate, thereby resulting in the formation of a dissociable enzyme.reagent complex comparable to that of a competitive inhibitor. Complex formation results in a high, localized concentration of reagent at the active site, thus increasing the likelihood of modification of a residue within this region as compared with modification of a like residue elsewhere in the protein molecule. In contrast to ideal general protein reagents which, due to their chemical nature, are selective for a given functional group, affinity labels are selective for a particular type of binding site and in many instances are reactive toward several functional groups found in proteins. The specificity with respect to enzyme is determined by the substratelike features of the reagent, and the specificity with respect to the kind of residue modified is a consequence of which reactive side chain within the active site is in proper juxtaposition to the leaving group of the reagent.

Reagents that we have synthesized as potential affinity labels for Rbl-P_2 carboxylase/oxygenase include Br-butanone-P_2, BrAcNHEtOP, cis and trans-2,3-epoxybutane-1,4-diol 1,4-bisphosphate, N-bromoacetyldiethanolamine bisphosphate, 2-phosphoglycolic acid azide, and N-bromoacetylphosphoserine. The routes of syntheses are illustrated in Figures 1-4. F-1-4

CRITERIA OF AFFINITY LABELING

At the outset, we emphasize that no single experiment can prove that enzyme inactivation reflects an active-site-directed modification. Rather, a variety of criteria must be met before a strong argument can be made with respect to active-site modification. It should also be remembered that proof of presence of a given residue at the active site does not prove that the residue plays a catalytic role. In fact, unequivocal proof of catalytic functionality cannot be provided by chemical modification alone.

Criteria that are useful in verifying affinity labeling include:

1. Complete Inactivation. If the residue subject to modification is absolutely essential to catalysis or substrate binding, total loss of enzymic activity should occur.

2. Pseudo-first Order Loss of Activity. At high molar ratios of reagent/enzyme, first order inactivation kinetics will be observed provided that inactivation correlates with the modification of one residue.

3. Rate Saturation. True affinity labels bind reversibly to the active site as an obligatory step preceding inactivation (equation 1). Thus, the rate of inactivation is proportional to the concentration of enzyme·reagent

$$E + I \underset{k_2}{\overset{k_1}{\rightleftharpoons}} E \cdot I \xrightarrow{k_3} E\text{-}I \qquad (1)$$

complex (E·I), and as the reagent concentration is increased the rate of inactivation will approach a limiting, finite value (rate saturation). A linear expression (equation 2) for the rate of inactivation has been derived[9,10]

$$\tau = (TK_{Inact} \cdot 1/[I]) + T \qquad (2)$$

in which K_{Inact} is $(k_2 + k_3)/k_1$, and comparable to K_M in the Michaelis-Menten expression, T is the minimal inactivation half-time, and τ is the observed inactivation half-time at inhibitor concentration [I]. From plots of τ vs. $1/[I]$, K_{Inact} and T are calculated.

4. Protection by Substrate (or Competitive Inhibitor). If the presumed affinity label binds reversibly to the active site preceding covalent modification, substrates should protect against inactivation in a competitive fashion (equation 3)[9,10]; i.e., the maximal rate of inactivation will be unaltered

$$\tau = (TK_{Inact}/I)\,(1 + [S]/K_S) + T \qquad (3)$$

with a decrease in apparent affinity (increased K_{Inact}) of reagent for enzyme. If the K_S (apparent dissociation constant for E·S) for protector (calculated from plots of τ vs. $1/[I]$) is similar to its K_M, a strong argument will have been made that the reagent initially binds reversibly to the active site.

5. Competitive Inhibition. Any reagent that inactivates an enzyme at a rate which is slow in comparison to the time necessary for enzyme assays, can be tested as a classical competitive inhibitor. If competitive inhibition is observed and the K_I proves to be equal to K_{Inact} calculated from inactivation kinetics, the enzyme·reagent complex that leads to inactivation must be the same complex that is visualized in the direct assays for inhibition.

6. Site-Specific Modification Requires Native 3D Structure. Since initial complexation with affinity label requires a functional substrate binding site, disruption of tertiary structure with a protein denaturant can eliminate the specificity in modification.

7. Stoichiometry. An ideal affinity label will be specific for a single site, and only one mole of reagent will be incorporated per mole of catalytic subunit inactivated.

8. Interspecies Homology Around Modified Residue. In general, active-site structure has been conserved during evolution. Thus, comparisons (at the level of amino acid sequences) of the reactions of a suspected affinity label with analogous enzymes from different species can demonstrate whether the site of modification represents a species invariant feature. The validity of this approach requires that the enzymes compared are derived from a common ancestral gene.

9. Pseudo Substrate. In some cases the affinity label so closely resembles the normal substrate that catalytic turnover of reagent occurs, thereby providing compelling documentation of active-site modification. An example is provided by 3-bromopyruvate, an affinity label for the aldolase that catalyzes condensation between pyruvate and D-glyceraldehyde 3-phosphate. Meloche et al.[11] demonstrated that the reagent undergoes enzyme-catalyzed proton exchange at C-3 as does the substrate pyruvate. The constant ratio (irrespective of bromopyruvate concentration) of moles of bromopyruvate undergoing proton exchange per mole of enzyme inactivated and the observation that the rates of both processes are half-saturated at identical reagent concentration strongly suggest that catalysis and alkylation occur at the same site.

AFFINITY LABELING STUDIES

General Comments

Affinity labeling studies on Rbl-P_2 carboxylase/oxygenase are complicated by the existence of distinct allosteric sites that bind phosphate esters[12] and by the conformational changes induced by Mg^{2+} and CO_2. Since the enzyme exists in an inactive conformation unless both Mg^{2+} and CO_2 are present[13,14], apparent inactivation by a chemical reagent could be caused by prevention of

the CO_2/Mg^{2+}-induced activation that normally takes place upon introduction of the inactive conformer into the assay medium. Thus, we have compared results obtained in the presence and absence of CO_2/Mg^{2+}. Such a comparison also provides an approach to distinguishing the catalytic site from the allosteric site, because the inactive conformer that exists in the absence of CO_2/Mg^{2+} is still functional in binding of phosphate esters[12].

Because of the complexities just stated, studies on carboxylases/oxygenases from different species may be necessary to confirm that a residue implicated as essential is indeed an active-site component. To provide an especially stringent test case for the identification of species invariant structural features, we have chosen for comparison the enzymes from two phylogenetically distant species, spinach and Rhodospirillum rubrum; the latter has the structurally simplest carboxylase/oxygenase known[15]. The structure and properties of the R. rubrum enzyme differ dramatically from those of the higher plant enzymes[15].

To date, the reactions of Br-butanone-P_2 and BrAcNHEtOP with the spinach enzyme have been rather thoroughly characterized[16-19]; only preliminary experiments have been completed with the R. rubrum enzyme[20]. Both enzymes were isolated by slight modification of published procedures[21,22] and assayed for carboxylase activity (spectrophotometrically[23] or $^{14}CO_2$-fixation[24]) and for oxygenase activity[24]. Syntheses for Br-butanone-P_2 and BrAcNHEtOP have been reported[25,26].

Reaction of Br-Butanone-P_2

Inactivation. Under conditions frequently used to assay Rbl-P_2 carboxylase/oxygenase (pH 8.0, room temperature), the spinach enzyme is inactivated by Br-butanone-P_2 (Figure 5). Rbl-P_2 protects against inactivation, and CO_2 greatly stimulates the rate of activity loss. Mg^{2+} does not influence F-5

the inactivation rate, and we assume therefore that it does not alter the sites of modification. Within the limits of the assay method, inactivation proceeds to completion. Activity is not regained upon exhaustive dialysis of the treated enzyme, thereby suggestive of covalent modification.

Competitive Inhibition. Reagent instability[25] has precluded a detailed kinetic study as would be necessary to demonstrate a rate saturation effect for the inactivation of the carboxylase/oxygenase by Br-butanone-P_2. However, the reagent is a competitive inhibitor (with respect to Rbl-P_2) of the enzyme from both spinach and R. rubrum with K_I's of 1.0 mM and 1.2 mM, respectively (Figure 6). F-6

Extent of Incorporation. The incorporation measured by reduction of the carbonyl group of the protein-bound reagent moiety with sodium [^{3}H]borohydride subsequent to protein derivatization with Br-butanone-P_2 is shown in Table 1. T-1 Also shown in the Table are the decreases in free sulfhydryl content that result during treatment with the reagent. Noteworthy is the observation that more extensive sulfhydryl alkylation results in the presence of substrate (without activity loss) than in its absence. Also, the number of free sulfhydryls lost in the presence of substrate is about the same as the total reagent incorporation. In contrast, the incorporation in the inactivated sample is clearly larger than the loss of sulfhydryls, and thus modification of residues other than cysteinyl must account for inactivation. Simple subtraction of the amount of reagent incorporated into the sample protected with Rbl-P_2 from the amount incorporated during inactivation indicates that inactivation correlates with modification of only 1.6 residues per native molecule (eight protomeric units). This value is misleadingly low because of the greater degree of modification of nonessential sulfhydryl groups that

occurs in the presence of Rbl-P_2. If, in the absence of Rbl-P_2, all residues modified other than sulfhydryls contribute to the inactivation process, loss of enzymic activity is due to derivatization of 5.8 residues (^3H incorporation minus sulfhydryl residues modified).

Kinds of Residues Modified. To determine which kind of residues other than cysteinyl are modified by Br-butanone-P_2, total acid hydrolysates of derivatized protein that was labeled with [^3H]borohydride were chromatographed on the amino acid analyzer (Figure 7). The elution positions of tritiated F-7 compounds were compared to those of authentic standards prepared by the reactions of glutathione and N-α-acetyllysine with Br-butanone-P_2 followed by reduction with [^3H]borohydride and finally acid hydrolysis. Most of the radioactivity from the substrate-protected sample elutes from the long column in the same positions (36 min and 42 min) as the cysteinyl derivatives prepared from glutathione[25]. In contrast, two radioactive peaks (that emerge ahead of lysine at 18 min and 24 min, respectively) are seen in the short column runs on hydrolysates of the inactivated enzyme which are not prominent in the protected samples. The first of these two peaks (the one at 18 min) is coincident with the product obtained from alkylation of acetyllysine by Br-butanone-P_2[25]. Although the second peak has not been completely characterized chemically, it too represents a lysyl derivative as will be shown.

The modification of lysyl residues must account for most of the inactivation, since this represents the major difference between inactivated and protected samples. This conclusion is supported by results of a differential labeling experiment (data not shown). The carboxylase/oxygenase was first treated with Br-butanone-P_2 under protective conditions, reduced with unlabeled borohydride, and dialyzed; the enzyme was then inactivated

by retreatment with Br-butanone-P_2 and reduced with [^{3}H]borohydride. Hydrolysates of this material contained the radioactive lysyl derivatives, but the cysteinyl derivatives were virtually absent.

Rbl-P_2 carboxylase/oxygenase from R. rubrum is also inactivated by Br-butanone-P_2, and the major difference between substrate-protected and inactivated enzyme is again in the level of lysyl derivatization[20].

Purification of Peptides Unique to Inactivated Carboxylase/Oxygenase and Identity of Modified Residues. Samples of inactivated and substrate-protected enzyme were digested with trypsin, and the digests were chromatographed on a cation exchange resin (Figure 8A). One of the major radioactive peaks seen in the digest of the inactivated enzyme is virtually absent in the digest of the substrate-protected enzyme. This peptide represents 3.3 residues per molecule of carboxylase. Except for this one radioactive peak, profiles from the two digests are quite similar; thus, the residues whose modifications result in inactivation must be represented by this peak. The peak unique to the inactivated carboxylase/oxygenase is resolved into two radioactive peptides upon chromatography on DEAE-cellulose (Figure 8B); the peptide eluting first is designated I and the other II. Peptides I and II are present in a ratio of about 1:2 and thus represent, respectively, 1.1 and 2.2 modified residues per molecule. After successive chromatography on phosphocellulose, Bio-Rad Aminex AG 1-X4, and Sephadex G-25, peptides I and II appear pure by peptide mapping and amino acid composition (Table 2). Hydrolysates of each peptide contain both radioactive derivatives that elute from the short column just ahead of lysine (Figure 9A). The sum of the two derivatives approximates one residue. Both derivatives represent lysyl residues as shown by treatment of the peptide hydrolysates with sodium metaperiodate followed by chromatography on the amino acid analyzer (Table 2, Figure 9B). After oxidation, the hydrolysates contain one additional

F-8

T-2

F-9

residue of lysine and lack both labeled derivatives. The radioactivity that was associated with the derivatives elutes with the front.

We believe that the presence of two distinct lysyl derivatives in seemingly pure peptides is a consequence of Br-butanone-P_2 reacting with lysyl residues to form two different products. Thus, peptides I and II could be mixtures of two peptides with identical structures with the exception of the chemical nature of a derivatized lysyl residue. This hypothesis is unproven.

Reaction of BrAcNHEtOP

Kinetics of Inactivation and Demonstration of Substrate Protection.

Incubation of spinach Rbl-P_2 carboxylase/oxygenase with BrAcNHEtOP results in a parallel loss of both activities in a pseudo-first order fashion (Figure 10). F-10 Apparent inactivation of the conformer that lacks enzymic activity (modifications carried out in the absence of CO_2/Mg^{2+}) occurs; Mg^{2+} alone stimulates the rate of inactivation; CO_2 alone decreases the rate of inactivation. This apparent effect of CO_2 is due to the increased ionic strength from added sodium bicarbonate, as the same effect is observed with sodium chloride. In combination, CO_2 and Mg^{2+} (conditions under which the enzyme is fully activated) result in a rate of inactivation by BrAcNHEtOP that is slightly lower than the rate seen in the presence of Mg^{2+} alone. Rbl-P_2 and the competitive inhibitor butane-1,4-diol 1, 4-bisphosphate[17] protect against inactivation.

A rate saturation is observed upon plotting the half-time of inactivation (τ) against the reciprocal reagent concentration (Figure 11). From these data, F-11 it is clear that Mg^{2+} increases the maximal velocity of inactivation (decreases T from 138 min to 24 min) and that $NaHCO_3$ merely alters the apparent affinity of BrAcNHEtOP for the enzyme. Without CO_2, the apparent dissociation constants

for the reagent·enzyme complexes (K_{Inact}) are 0.8 mM and 3.0 mM in the absence and presence of Mg^{2+}, respectively. Thus, Mg^{2+} alters both T and K_{Inact}. In the presence of 66 mM $NaHCO_3$, K_{Inact} increases to 2.9 mM and 11 mM without and with Mg^{2+}, respectively.

Extent of Incorporation. With [^{14}C]BrAcNHEtOP, the degree of protein modification is determined. The enzyme inactivated in the absence of Mg^{2+} contains about 2.7 moles of reagent per mole of protomer; a corresponding sample protected by Rbl-P_2 contains about 0.9 mole of reagent. The presence of Mg^{2+} in the modification reaction mixture reduces the level of incorporation to about 1.4 moles of reagent per mole of protein, and the corresponding sample protected by butanediolbisphosphate contains about 0.5 mole of reagent. By determining the level of incorporation during the time-course of the incubation with reagent, a direct proportionality is seen between loss of enzymic activity and incorporation (expressed as the difference in incorporation between unprotected and protected samples) (Figure 12). Extrapolation of these data F-12
to complete inactivation gives values of 1.8 moles of reagent per mole of protomer in the absence of Mg^{2+} and 1.2 moles of reagent in the presence of Mg^{2+}.

Kinds of Residues Modified. The sites of reaction of [^{14}C]BrAcNHEtOP are readily determined by amino acid analysis, since the reagent is an N-substituted carboxamidomethyl compound and thus all derivatized residues will appear in acid hydrolysates as carboxymethyl amino acids. Radioactivity profiles from the amino acid analyzer for hydrolysates of carboxylase/oxygenase modified in the presence and absence of Mg^{2+} are compared in Figure 13. CO_2 F-13
was present during all modifications so that the differences observed must reflect the Mg^{2+}-induced conversion of enzymically inactive conformer to the

active one. Carboxymethylcysteine is the major radioactive compound found in hydrolysates of enzyme that was modified in the absence of Mg^{2+}. The corresponding substrate-protected sample contains one less residue of carboxymethylcysteine per protomer. In addition to the cysteinyl derivative, carboxymethyllysine is found in hydrolysates of carboxylase/oxygenase after modification in the presence of Mg^{2+}. Butanediolbisphosphate gives slight protection of cysteinyl residues (0.2 per protomer) but essentially complete protection of lysyl residues (0.9 per protomer). Thus, inactivation correlates with cysteinyl or lysyl alkylation depending upon the absence or present of Mg^{2+}, respectively.

Purification of Peptides Unique to Inactivated Carboxylase/Oxygenase. Tryptic digests of modified enzyme were subjected to ion exchange chromatography on Bio·Rad Aminex AG 50 W-X2. Two major radioactive peptides (designated C1 and C2, which on the basis of radioactivity represent 0.75 and 0.46 moles of peptide per mole of inactive protomer, respectively) are resolved from tryptic digests of enzyme inactivated in the absence of Mg^{2+}; these peptides are missing from digests of substrate-protected enzyme (Figure 14A). In contrast, F-14 enzyme inactivated in the presence of Mg^{2+} yields a single major radioactive tryptic peptide (designated L1, and representing 0.73 mol of peptide per mol of inactive protomer) that is not seen in samples protected by butanediol-bisphosphate or the transition-state analog carboxyribitolbisphosphate[27] (Figure 14B). To determine the type of residue modified in the labeled peptides, an aliquot of each peak was subjected to amino acid analysis; these identifications are indicated in the figure. Both of the two major substrate-protected radioactive peptides in Figure 14A (from enzyme inactivated in the absence of Mg^{2+}) are derivatized at cysteinyl residues, while the major radioactive peptide in Figure 14B (from enzyme inactivated in the presence of Mg^{2+})

is derivatized at a lysyl residue. Peptides C1, C2, and L1 were purified to homogeneity by successive chromatography on DEAE cellulose and phosphocellulose, and their amino acid compositions are given in Table 3. As T-3 expected, hydrolysates of peptides C1 and C2 contain one radioactive Cys(Cm) residue, and the hydrolysate of peptide L1 contains one radioactive Lys(ε-Cm) residue. The latter also contains one Cys(Cm) residue; this, however, is not radioactive and is therefore not reagent-derived (preceding tryptic digestion, all free -SH groups in the enzyme were carboxymethylated with iodoacetate). Peptides C2 and L1 have identical compositions (with the exception of the residue that bears the reagent moiety), which are the same as the composition of peptide I obtained from the enzyme derivatized by Br-butanone-P_2 (see Table 2).

Sequence Determinations

Peptides I and II obtained from carboxylase/oxygenase modified by Br-butanone-P_2 and peptides C1, C2, and L1 obtained from the enzyme modified by BrAcNHEtOP were sequenced by automated Edman degradations in a Beckman 890C Sequencer. The established sequences follow (the residues illustrated in boldface carry the reagent moiety):

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

I Tyr-Gly-Arg-Pro-Leu-Leu-Gly-Cys-Thr-Ile-**Lys**-Pro-Lys

L1 Tyr-Gly-Arg-Pro-Leu-Leu-Gly-Cys-Thr-Ile-**Lys**-Pro-Lys

C2 Tyr-Gly-Arg-Pro-Leu-Leu-Gly-**Cys**-Thr-Ile-Lys-Pro-Lys

C1 Trp-Ser-Pro-Glu-Leu-Ala-Ala-Ala-**Cys**-Glu-Val-Trp-Lys

As anticipated from their amino acid compositions, peptides I, L1, and C2 are derived from the same region of the polypeptide chain and differ only in the nature and site of derivatization.

Other Potential Affinity Labels

The cis- and trans-epoxybutanediolbisphosphates were synthesized in an effort to find a reagent with binding specificity similar to that of Br-butanone-P_2 but with decreased chemical reactivity so as to minimize the modification of nonessential sulfhydryl groups. The rationale for preparing N-bromoacetyldiethanolaminebisphosphate was that by extending the distance between the two phosphate groups as found in Br-butanone-P_2 to more closely approximate Rbl-P_2 an increased affinity for the active site would result. N-Bromoacetylphosphoserine and phosphoglycolic acid azide represent attempts to utilize reactive analogs of 3-phosphoglycerate and phosphoglycolate as affinity labels. None of these reagents inactive Rbl-P_2 carboxylase/oxygenase at a sufficiently rapid rate so as to merit further experimentation.

CONCLUSIONS

Despite the alkylation of nonessential sulfhydryls and the incomplete characterization of the lysyl derivatives, inactivation of spinach Rbl-P_2 carboxylase/oxygenase by Br-butanone-P_2 is clearly a consequence of modification of two different lysyl residues. Based on the stoichiometry of lysyl modification (the combined yield of peptides I and II is less than one mole per mole of protomer) and the constant ratio of peptide I/peptide II irrespective of Br-butanone-P_2 concentration used to inactivate (data not shown), we believe that within a given subunit the two lysyl residues are mutually exclusive with respect to modification and that modification of either is sufficient for inactivation. The affinity of Br-butanone-P_2 for Rbl-P_2 carboxylase/oxygenase as demonstrated by the observed competitive inhibition and the protection afforded by Rbl-P_2 against inactivation suggest that the two labeled lysyl residues are in the active-site region. Other observations that indirectly support this conclusion are the unusual

reactivity of the lysyl residues modified (in model systems the reactivity of Br-butanone-P_2 toward sulfhydryls exceeds its reactivity toward amino groups by at least 100-fold[25]), the lack of reactivity of these lysyl residues in denatured enzyme[28] (i.e., the selective modification of lysyl residues requires a catalytically functional binding site), the presence of these lysyl residues within the large subunit as is the catalytic site[29], and the inactivation of R. rubrum Rbl-P_2 carboxylase/oxygenase as a consequence of lysyl alkylation.

As regards the inactivation of Rbl-P_2 carboxylase/oxygenase by BrAcNHEtOP, the lysyl residue susceptible to alkylation in the presence of CO_2/Mg^{2+} almost certainly occupies a position within the active-site region. The degree of inactivation is directly proportional to the extent of inactivation; the lysyl residue is protected by a competitive inhibitor and the transition-state analog against alkylation; and inactivation exhibits rate saturation, suggesting specific binding of reagent as a prerequisite to covalent reaction. Alkylation of a single lysyl residue represents a high degree of specificity (each protomer contains thirty lysines[30]), and, as with Br-butanone-P_2, represents an unusual reactivity in comparison to model compounds in which sulfhydryl groups react far more rapidly than amino groups. Indirect evidence that BrAcNHEtOP reacts at a site for Rbl-P_2 is provided by the finding that the reagent binds tighter to the inactive conformer (K_{Inact} of 2.9 mM in the absence of Mg^{2+}) than to the active conformer (K_{Inact} of 11 mM in the presence of Mg^{2+}) as was observed for substrate[12,31].

The inactivation that occurs in the absence of Mg^{2+} and that correlates with modification of two different sulfhydryl groups might be a consequence of prevention of the CO_2/Mg^{2+}-induced activation. However, this inactivation

also exhibits rate saturation and is prevented by Rbl-P_2 as a consequence of protection against modification.

Sequence determinations of the tryptic peptides containing the essential residues have proven quite informative. We find that Br-butanone-P_2 and BrAcNHEtOP have one common site of reaction (see sequences of peptides I and L1). Thus, two chemically-different, reactive phosphate esters with demonstrated affinities for the carboxylase/oxygenase alkylate the same lysyl residue. This observation, taken together with data already discussed, provides a rather compelling argument that the lysyl residue in question is within a binding site for phosphate esters.

The sequence data also suggest that the catalytic site and the previously proposed[12] allosteric site for Rbl-P_2 are equivalent, overlapping, or contiguous. The presence of the allosteric site is inferred primarily from the knowledge that Rbl-P_2 binds tenaciously to the inactive conformer and inhibits activation by Mg^{2+}/CO_2[12-14]. These observations, however, do not rule out the existence of only a single site with nonproductive binding to the inactive conformer, as suggested earlier[4,14]. In agreement with this possibility, we find that in the absence of Mg^{2+} (inactive conformer) inactivation correlates with the modification of two cysteinyl residues, one of which is only three residues removed from the lysyl residue that is selectively modified in the presence of Mg^{2+} (see sequences of peptides L1 and C2). Thus, it seems plausible that Mg^{2+} alters slightly the topology of a single binding site for Rbl-P_2 (or BrAcNHEtOP) so that in the reagent-enzyme complex formed from inactive conformer a sulfhydryl is accessible for alkylation, whereas in the reagent-enzyme complex formed from active conformer an ε-amino group is accessible. Also consistent with equivalence of the presumed allosteric site for Rbl-P_2 and the catalytic site is the fact that

the lysyl residue accessible to both reagents is alkylated by BrAcNHEtOP in the presence of Mg^{2+} but alkylated by Br-butanone-P_2 in the absence of Mg^{2+}.

The finding of at least one cysteinyl residue in the region of Rbl-P_2 binding may explain, in part, the previously recognized sensitivity of the enzyme to sulfhydryl reagents.

Studies on the inactivation of Rbl-P_2 carboxylase/oxygenase by pyridoxal phosphate are also consistent with the presence of lysyl residues at the active site. The reagent is highly selective in that inactivation correlates with the modification of a small number of lysyl residues (0.5-1.0/protomer)[4-6]. Based on inhibition studies[4], pyridoxal phosphate has a high affinity for the spinach enzyme; complex formation between the R. rubrum enzyme and reagent is indicated by saturation kinetics of inactivation[6]. Thus, several types of data suggest that pyridoxal phosphate is an active-site-directed reagent for carboxylase/oxygenase. It will be of interest to learn whether the lysyl residue(s) modified is the same as one of those modified by Br-butanone-P_2 or BrAcNHEtOP.

The amino group of spinach carboxylase/oxygenase that is modified by pyridoxal phosphate has been suggested as the site for binding of CO_2 as substrate[4]. This suggestion was prompted by the finding of inhibition (with respect to CO_2) of the enzyme by pyridoxal phosphate. However, whether the inhibition is competitive or noncompetitive is unclear. When pyridoxal phosphate was tested by identical methodologies under the same conditions with either CO_2 or Rbl-P_2 as the variable substrate, inhibition appeared noncompetitive in both cases. Furthermore, the lack of protection against inactivation afforded by saturating levels of bicarbonate seems inconsistent with an involvement of the target lysyl residue in CO_2 binding. Another perplexing result with the spinach enzyme is the absence of a

rate saturation for inactivation despite a K_I of 1 μM for pyridoxal phosphate in the inhibition experiments.

Since bicarbonate stimulates the rate of inactivation of spinach carboxylase/oxygenase by Br-butanone-P_2, the two lysyl residues that are sites of modification (one of these residues is also modified by BrAcNHEtOP) do not appear to be involved in binding of CO_2 either as effector or as substrate. We believe that interpretations of existing chemical modification data in terms of precise catalytic roles of the residues labeled are premature.

Acknowledgments. Research sponsored by the Division of Biological and Environmental Research, U.S. Department of Energy under contract W-7405-eng-26 with the Union Carbide Corporation. JVS is a predoctoral fellow supported by Grant GM 1974 from the National Institute of General Medical Sciences, National Institutes of Health.

REFERENCES

1. NISHIMURA, M., AND AKAZAWA, T. (1974) J. Biochem. 76, 169-76.
2. TAKABE, T., AND AKAZAWA, T. (1975) Arch. Biochem. Biophys. 169, 686-94.
3. SUGIYAMA, T., AKAZAWA, T., NAKAYAMA, N., AND TANAKA, Y. (1968) Arch. Biochem. Biophys. 125, 107-13.
4. PAECH, C., RYAN, F.J., AND TOLBERT, N.E. (1977) Arch. Biochem. Biophys. 179, 279-88.
5. WHITMAN, W.B., AND TABITA, F.R. (1978) Biochemistry 17, 1282-87.
6. WHITMAN, W.B., AND TABITA, F.R. (1978) Biochemistry 17, 1288-93.
7. LAWLIS, V.B., AND McFADDEN, B.A. (1978) Biochem. Biophys. Res. Commun. 80, 580-85.
8. SCHLOSS, J.V., NORTON, I.L., STRINGER, C.D., AND HARTMAN, F.C. (1978) Federation Proc. 37, 1310.
9. KITZ, R., AND WILSON, I.B. (1962) J. Biol. Chem. 237, 3245-49.
10. MELOCHE, H.P. (1967) Biochemistry 6, 2273-80.
11. MELOCHE, H.P., LUCZAK, M.A., AND WURSTER, J.M. (1972) J. Biol. Chem. 247, 4186-91.
12. CHU, D.K., AND BASSHAM, J.A. (1975) Plant Physiol. 55, 720-26.
13. LORIMER, G.H., BADGER, M.R., AND ANDREWS, T.J. (1976) Biochemistry 15, 529-36.
14. LAING, W.A., AND CHRISTELLER, J.T. (1976) Biochem. J. 159, 563-70.
15. McFADDEN, B.A., AND TABITA, F.R. (1974) BioSystems 6, 93-112.
16. NORTON, I.L., WELCH, M.H., AND HARTMAN, F.C. (1975) J. Biol. Chem. 250, 8062-68.
17. SCHLOSS, J.V., AND HARTMAN, F.C. (1977) Biochem. Biophys. Res. Commun. 77, 230-35.
18. STRINGER, C.D., AND HARTMAN, F.C. (1978) Biochem. Biophys. Res. Commun. 80, 1043-48.
19. SCHLOSS, J.V., STRINGER, C.D., AND HARTMAN, F.C. (1978) J. Biol. Chem. (in press).
20. SCHLOSS, J.V., AND HARTMAN, F.C. (1977) Biochem. Biophys. Res. Commun. 75, 320-28.
21. WISHNICK, M., AND LANE, M.D. (1971) Methods Enzymol. 23, 570-77.

22. TABITA, F.R., AND McFADDEN, B.A. (1974) J. Biol. Chem. 249, 3453-58.

23. RACKER, E. (1963) in Methods of Enzymatic Analysis, pp. 188-90, H. U. Bergmeyer, Editor, Academic Press, New York.

24. LORIMER, G.H., BADGER, M.R., AND ANDREWS, T.J. (1977) Anal. Biochem. 78, 66-75.

25. HARTMAN, F.C. (1975) J. Org. Chem. 40, 2638-42.

26. HARTMAN, F.C., SUH, B., WELCH, M.H., AND BARKER, R. (1973) J. Biol. Chem. 248, 8233-39.

27. SIEGEL, M.I., AND LANE, M.D. (1972) Biochem. Biophys. Res. Commun. 48, 508-16.

28. HARTMAN, F.C., WELCH, M.H., AND NORTON, I.L. (1973) Proc. Natl. Acad. Sci. USA 70, 3721-24.

29. NISHIMURA, M., AND AKAZAWA, T. (1973) Biochem. Biophys. Res. Commun. 54, 842-48.

30. SIEGEL, M.I., WISHNICK, M., AND LANE, M.D. (1972) in The Enzymes, 3rd Ed, Vol. 6, p. 177, P.D. Boyer, Editor, Academic Press, New York.

31. WISHNICK, M., LANE, M.D., AND SCRUTTON, M.C. (1970) J. Biol. Chem. 245, 4939-47.

TABLE 1

Extent of incorporation of Br-butanone-P_2 into Rbl-P_2 carboxylase and number of sulfhydryl groups modified

Sample	Enzymatic activity	^3H	Number of residues of carboxymethyl cysteine*	Number of sulfhydryl groups modified
	% remaining	mol reagent/ mol enzyme		
Inactivated	1	13.8	90	8
Substrate-protected	95	12.2	87	11
Control	100		98	

*Determined by amino acid analysis after carboxymethylation of the protein sample with iodoacetate.

TABLE 2

Amino acid compositions of purified peptides containing essential residues of Rbl-P_2 carboxylase that are modified by Br-butanone-P_2

Amino acid	Number of residues			
	I	II	After periodate oxidation	
			I	II
Derivative (18 min)*	0.5	0.6		
Derivative (24 min)*	0.7	0.7		
Lysine	1.0		2.1	1.0
Histidine		2.0		1.7
Arginine	1.1	1.0	1.0	1.0
Carboxymethylcysteine	1.0		0.6	
Aspartic acid		1.0		1.1
Threonine	1.0	1.0		
Serine		2.0		
Glutamic acid		2.1		2.2
Proline	1.9		1.8	
Glycine	1.9	5.2	2.2	4.9
Alanine				
Valine		2.1		2.1
Methionine				
Isoleucine**	1.0	1.0	1.0	1.0
Leucine	2.0	2.0	2.0	2.0
Phenylalanine				
Tyrosine	1.0		0.4	

*Time referes to elution position of the derivative from the short column of the amino acid analyzer.

**The quantity of isoleucine in the sample was arbitrarily set to 1.0 residue.

TABLE 3

Amino acid compositions of peptides unique to BrAcNHEtOP-inactivated Rbl-P_2 carboxylase/oxygenase

Amino acid	Number of residues		
	C1	C2	L1
Lysine	0.9	2.0	1.0
ε-carboxymethyllysine			1.0*
Arginine		1.1	1.0
Tryptophan**	2.2		
Carboxymethylcysteine	0.9*	1.0*	0.9
Threonine		0.9	1.0
Serine	0.9		
Glutamic Acid	2.1		
Proline	0.9	1.9	1.9
Glycine		2.0	2.2
Alanine	2.9		
Valine	0.9		
Isoleucine		1.0***	1.0***
Leucine	1.0***	2.1	2.1
Tyrosine		0.9	1.0

*Based on assays for radioactivity in the effluent from the amino acid analyzer, this residue is labeled and therefore bears the reagent moiety in the intact peptide.

**Trp was determined from the A_{280nm}.

***This amino acid was arbitrarily assigned a value of 1.0.

FIGURE LEGENDS

Figure 1. Synthesis of Br-butanone-P_2.

Figure 2. Syntheses of BrAcNHEtOP, bromoacetylphosphoserine, and bromoacetyldiethanolamine bisphosphate.

Figure 3. Syntheses of cis- and trans- 2,3-epoxybutane-1,4-diol 1,4-bisphosphate.

Figure 4. Synthesis of phosphoglycolic acid azide.

Figure 5. Loss of carboxylase activity upon incubation of Rbl-P_2 carboxylase/oxygenase (1 mg/ml) with Br-butanone-P_2 (0.1 mM) in 0.1 M Bicine/60 mM $KHCO_3$/1 mM EDTA (pH 8.0) (●). In other experiments, Rbl-P_2 (1 mM) was added to the buffer (▲) or bicarbonate was omitted (o).

Figure 6. Lineweaver-Burk plots for the inhibition of spinach (●,o) and R. rubrum (▲,Δ) carboxylases by Br-butanone-P_2. Prior to assay, enzyme was incubated in 10 mM $MgCl_2$/66 mM $NaHCO_3$/50 mM Bicine (pH 8.0). Assays were carried out during a 60-second interval with the $^{14}CO_2$-fixation method[24] in the absence (▲,●) or presence (Δ,o) of 2 mM inhibitor.

Figure 7. Radioactivity in hydrolysates of carboxylase/oxygenase after treatment, in the absence (——) and presence (---) of Rbl-P_2, with Br-butanone-P_2 followed by reduction with sodium [^{3}H]borohydride. Hydrolysates were chromatographed on the amino acid analyzer, and 1-min fractions of the effluents, after their emergence from the flow cell, were collected and counted. The absorbance at 570 nm is not shown, but the elution positions of some amino acids are indicated.

Figure 8. Chromatography on Bio-Rad AG 50W-X2 of tryptic digests of carboxylase/oxygenase after treatment with Br-butanone-P_2 in the absence (——) and presence (---) of Rbl-P_2 (A). The major peak that is unique to the inactivated enzyme (fractions 50-57 in A) was chromatographed on DEAE-cellulose (B).

Figure 9. Analyses, on the short column of the amino acid analyzer, of hydrolysates of peptides I and II before (A) and after (B) oxidation with sodium metaperiodate.

Figure 10. Time-course of inactivation of Rbl-P_2 carboxylase/oxygenase (10 mg/ml) by 5 mM BrAcNHEtOP in 50 mM Bicine buffer (pH 8.0) with no additions (●), 5 mM $MgCl_2$ (▲), 66 mM $NaHCO_3$ (o), 5 mM $MgCl_2$/66 mM $NaHCO_3$ (Δ).

Figure 11. Inactivation half-time (τ) of Rbl-P_2 carboxylase as a function of the reciprocal of BrAcNHEtOP concentration. The following conditions were used: 5 mM $MgCl_2$/50 mM bicine (Δ); 5 mM $MgCl_2$/66 mM $NaHCO_3$/50 mM Bicine (▲); Mg^{2+}-free 50 mM bicine (0); Mg^{2+}-free 66 mM $NaHCO_3$/50 mM Bicine (●). All buffers were pH 8.0.

Figure 12. Incorporation of [^{14}C]BrAcNHEtOP as a function of enzymic activity lost in the absence (□) and presence (0) of Mg^{2+}. Incorporation is expressed as the difference in incorporation between unprotected enzyme and protected enzyme (Rbl-P_2 is the protector without Mg^{2+} and butanediol-P_2 the protector with Mg^{2+}).

Figure 13. Chromatographic profiles of hydrolysates of carboxylase/oxygenase after modification with [^{14}C]BrAcNHEtOP. Chromatography was accomplished with an amino acid analyzer without use of its ninhydrin system; 2-min fractions were collected and counted. Profiles shown in the upper panel are for enzyme samples modified in the absence of Mg^{2+} with (---) or without (——) protector (Rbl-P_2). Profiles shown in the lower panel are for enzyme samples modified in the presence of Mg^{2+} with (---) or without (——) protector (butanediol-P_2).

Figure 14. Chromatography on BioRad AG 50 of tryptic digests of enzyme modified by [^{14}C]BrAcNHEtOP. The kind of labeled carboxymethyl amino acid found in hydrolysates of each major radioactive peptide is indicated in the figure. (A) The enzyme was modified in the absence of Mg^{2+} with (---) or without (——) protector (1 mM Rbl-P_2). (B) The enzyme was modified in the presence of Mg^{2+} without protector (——), with 20 mM butanediol-P_2 (---), or with 0.2 mM carboxyribitolbisphosphate (....).

27281-1

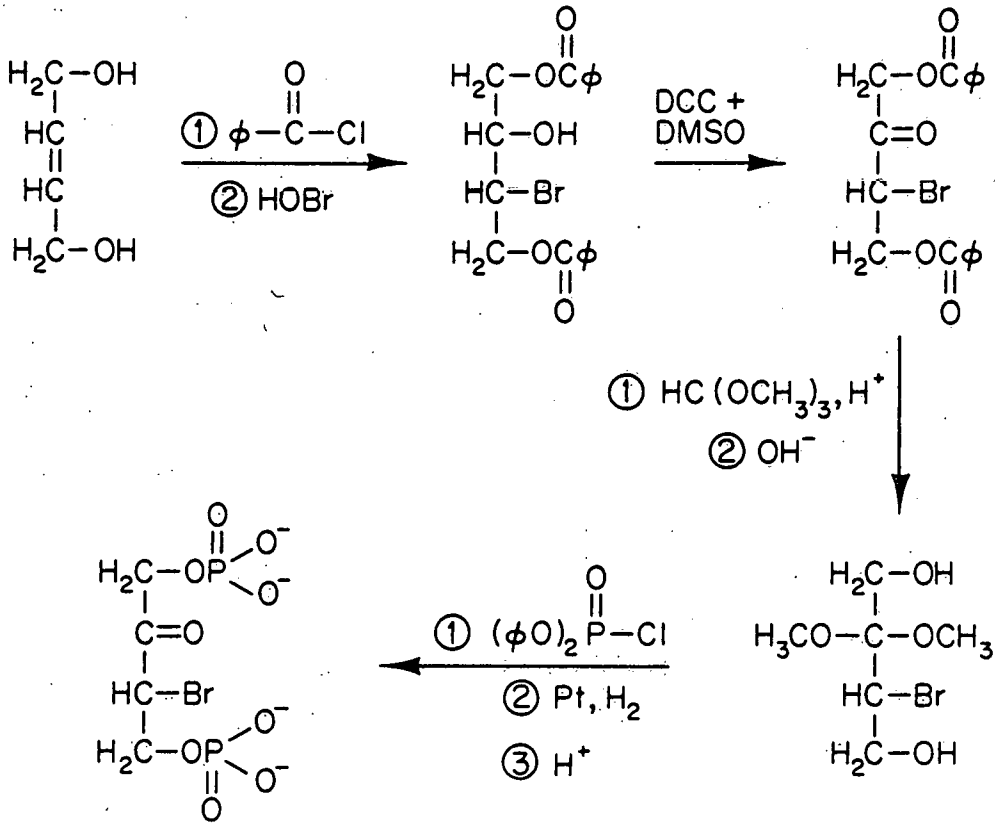

Fig. 1

34784

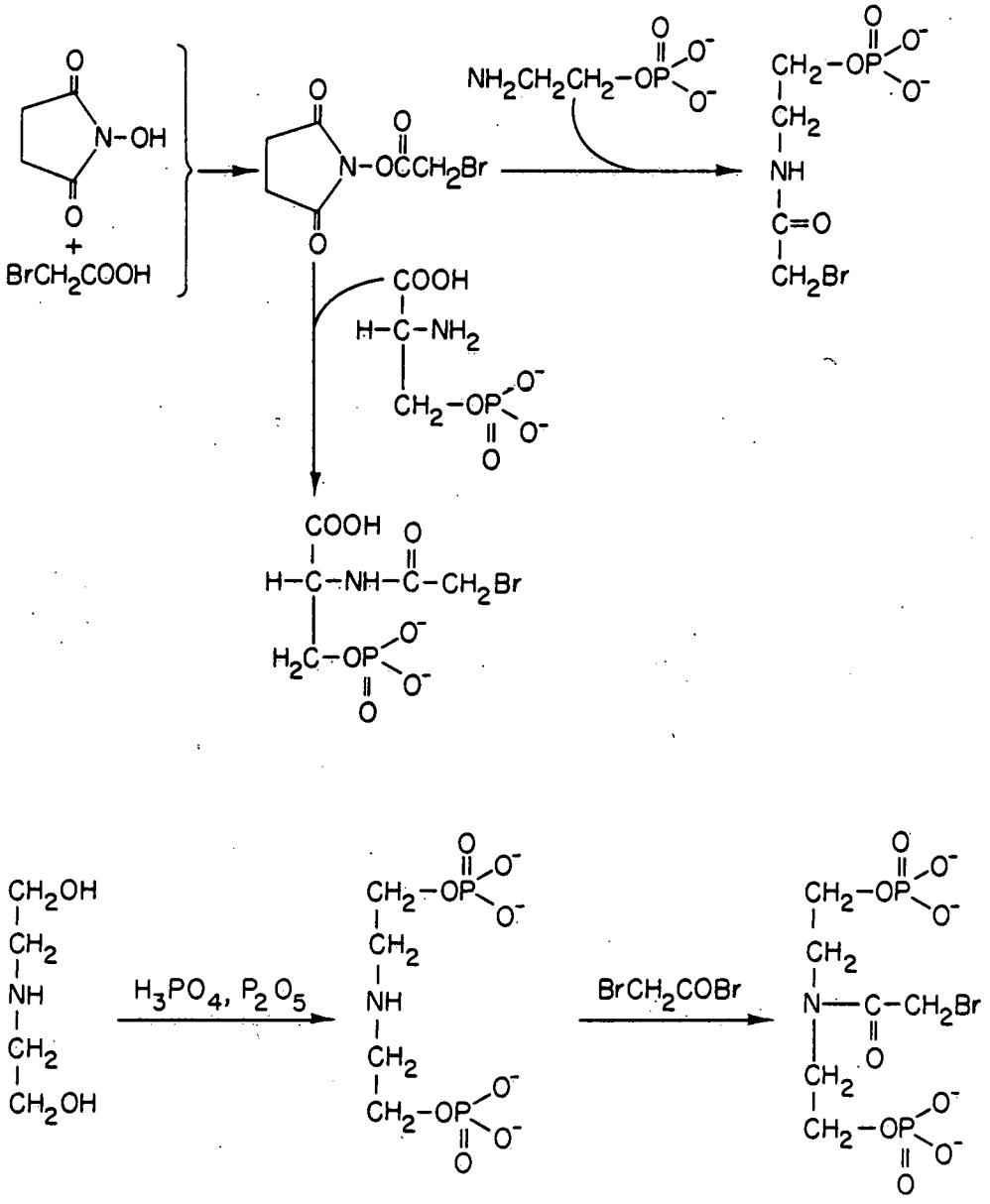
N-OH
+
BrCH2COOH
N-OCCH2Br
NH2CH2CH2-OP
CH2-OP
CH2
NH
C=O
CH2Br
COOH
H-C-NH2
CH2-OP
COOH
H-C-NH-C-CH2Br
H2C-OP
CH2OH
CH2
NH
CH2
CH2OH
H3PO4, P2O5
BrCH2COBr
N-C-CH2Br

Fig. 2

33702-1

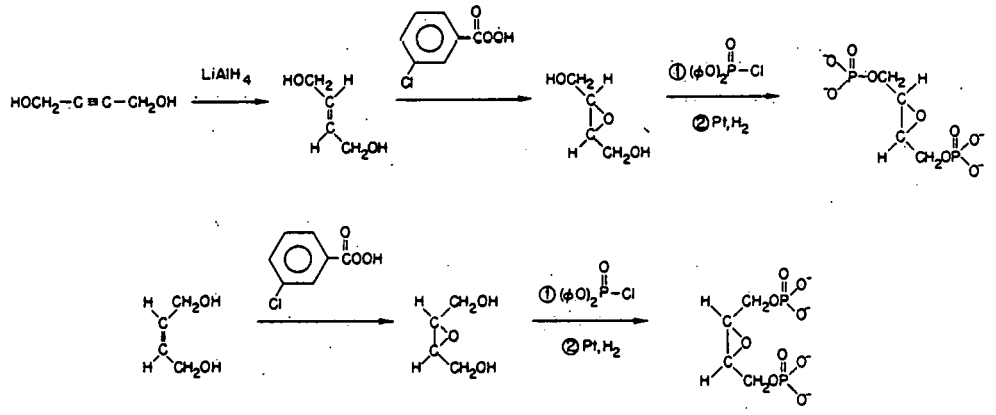
HOCH2–C≡C–CH2OH
LiAlH4
HOCH2 H
CH2OH
COOH
Cl
HOCH2 H
CH2OH
① (φO)2P–Cl
② Pt, H2
H CH2OH
CH2OH
COOH
Cl
H CH2OH
CH2OH
① (φO)2P–Cl
② Pt, H2

Fig 3

34789-1

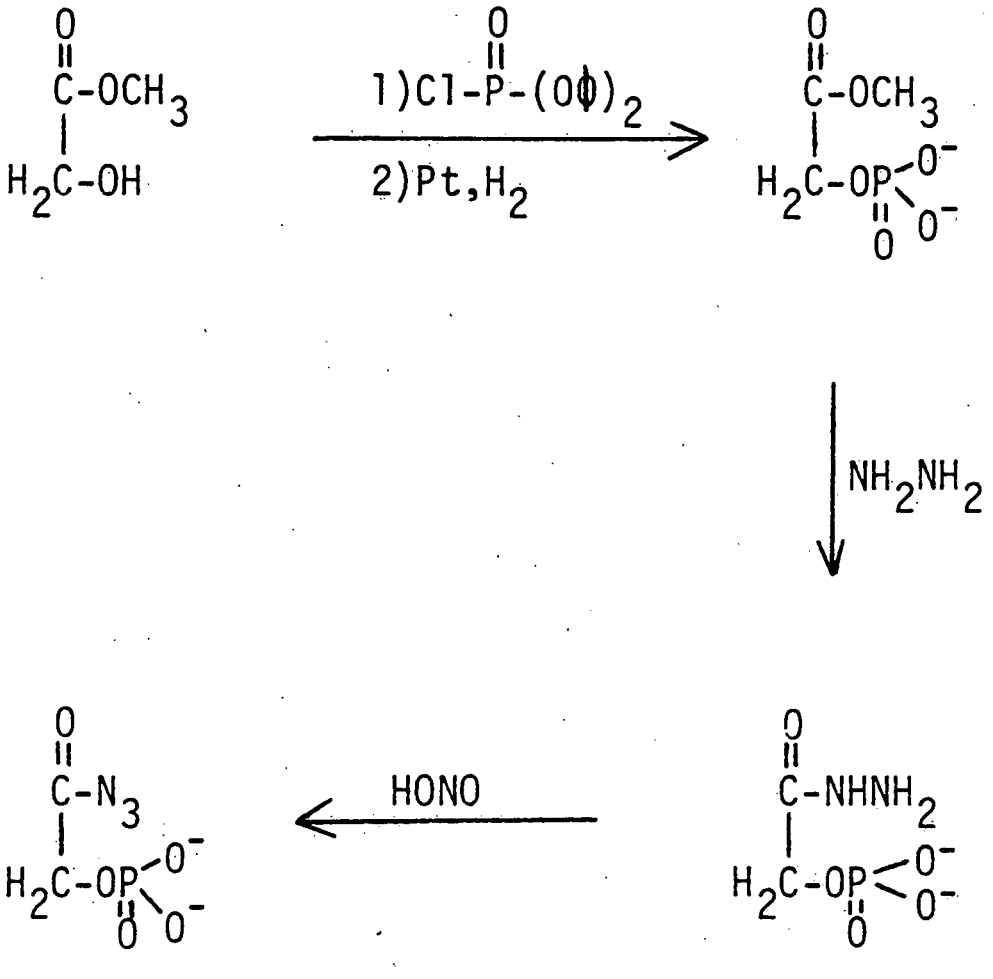

Fig 4

27591-1

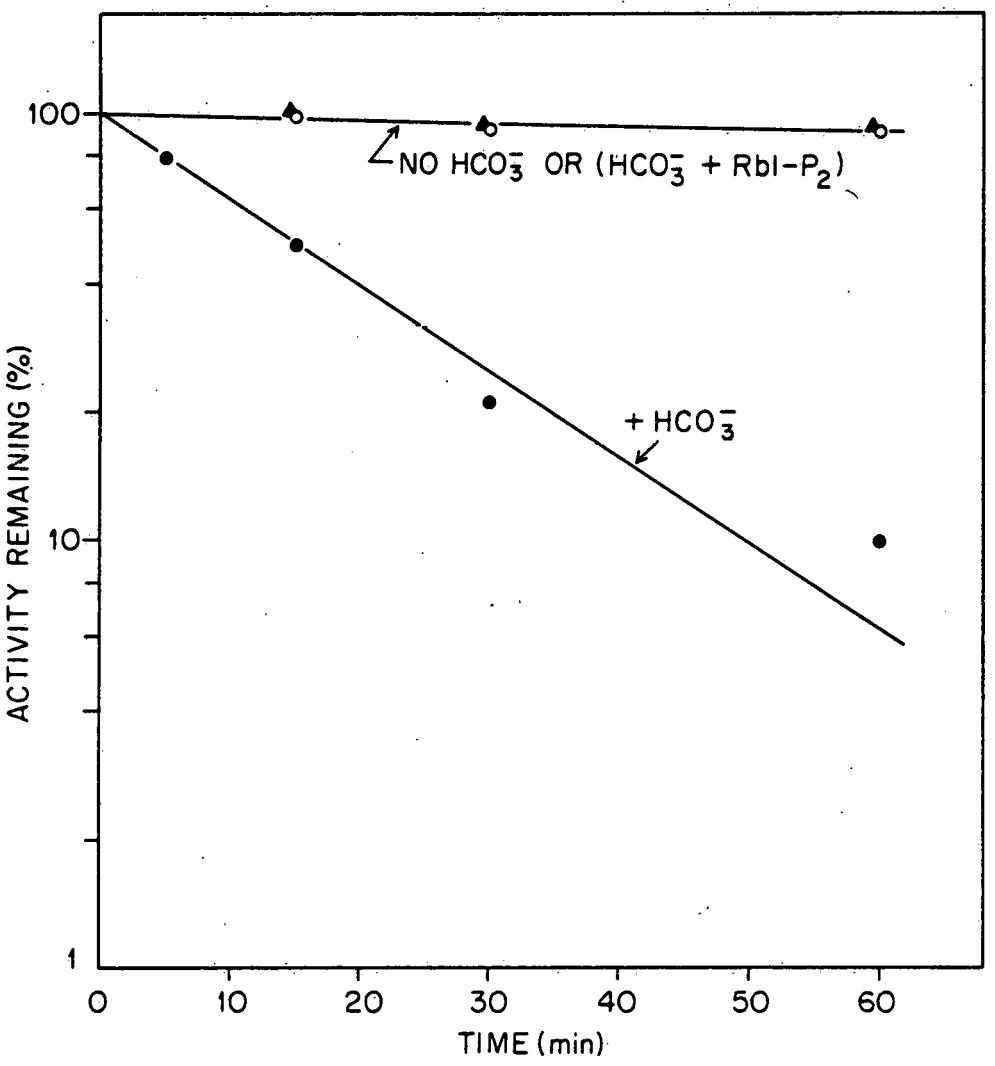
100
10
1
ACTIVITY REMAINING (%)
NO HCO_3^- OR (HCO_3^- + Rbl-P_2)
+ HCO_3^-
0
10
20
30
40
50
60
TIME (min)

Fig. 5

34786

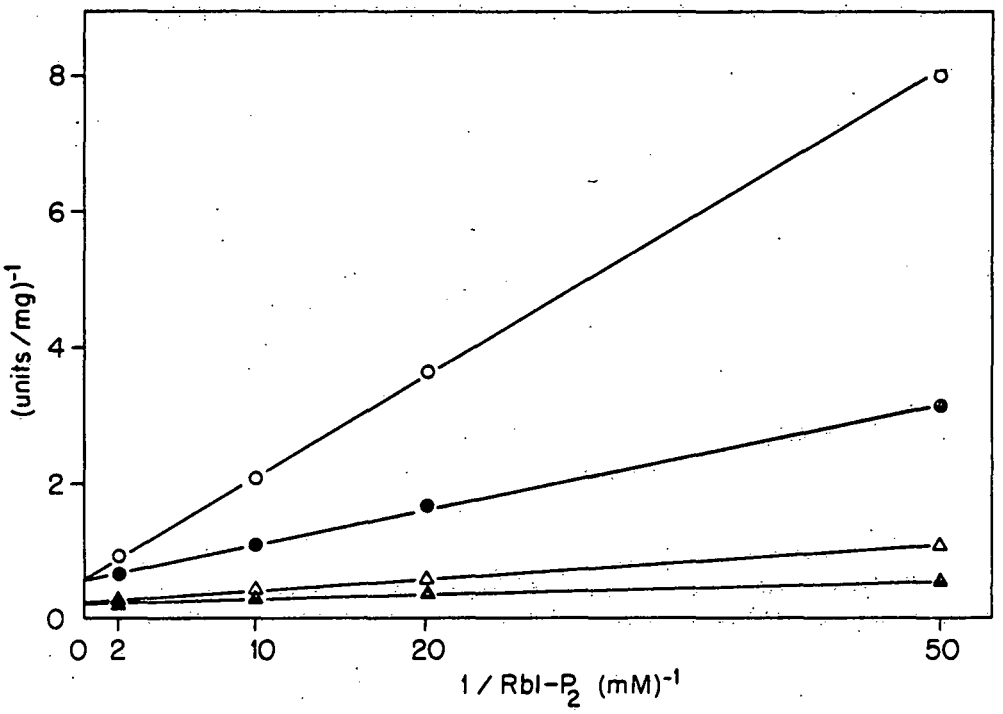

Fig. 6

30238

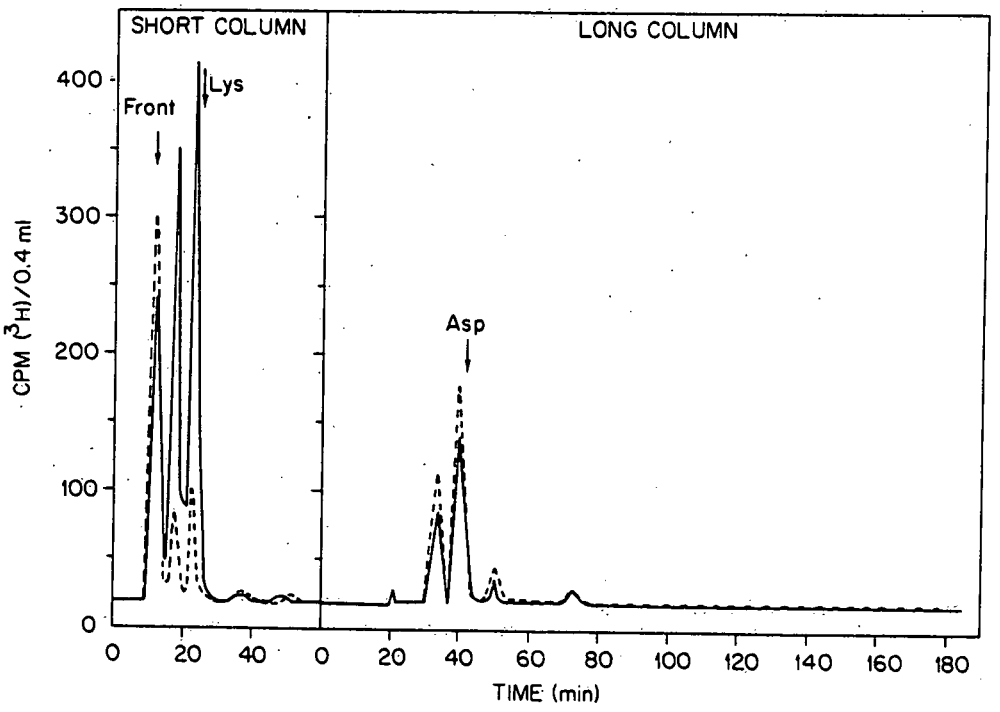
SHORT COLUMN
LONG COLUMN
Front
Lys
Asp
CPM (^{3}H)/0.4 ml
400
300
200
100
0
0
20
40
0
20
40
60
80
100
120
140
160
180
TIME (min)

Fig 7

29310

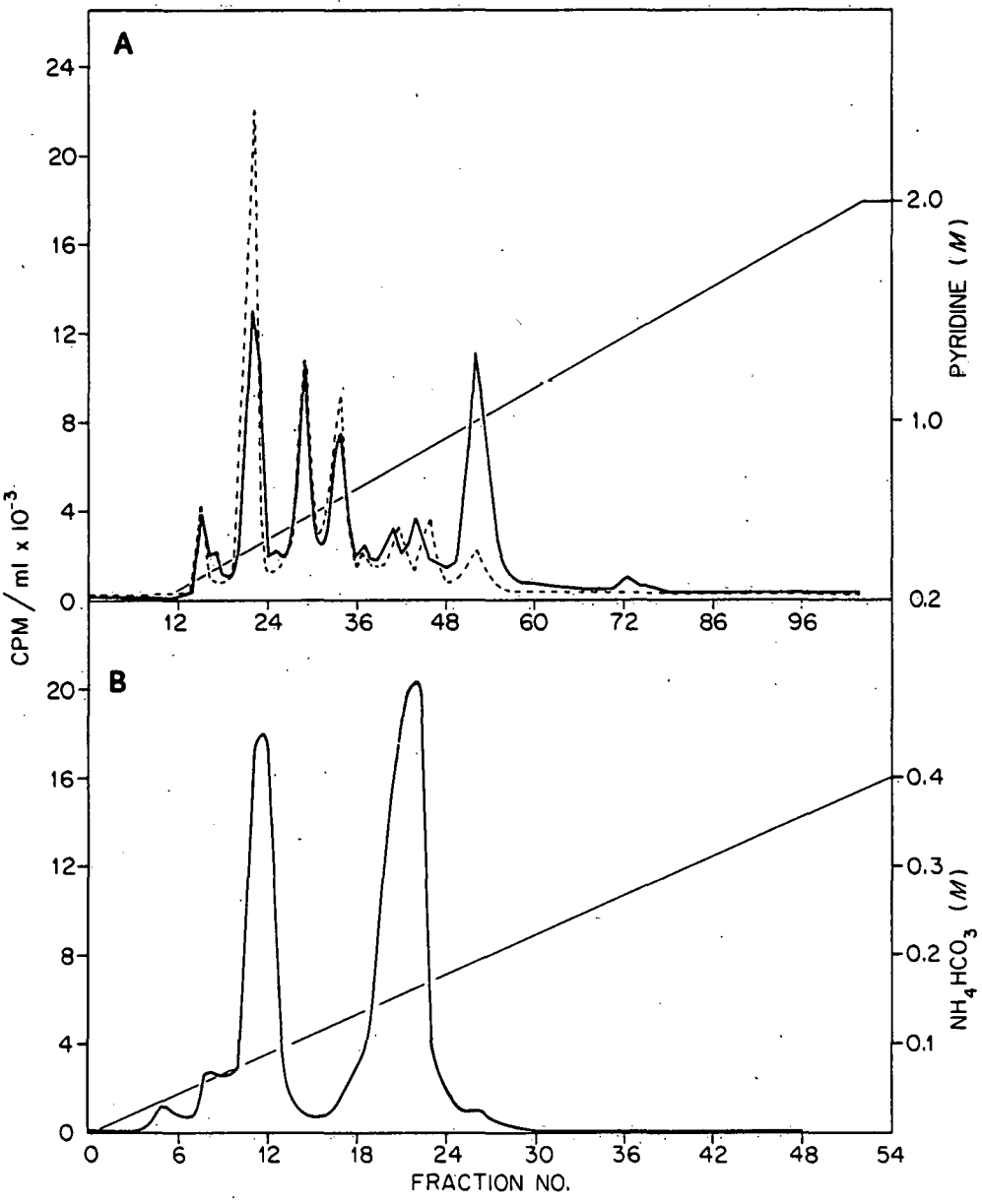
A
B
CPM / ml x 10^{-3}
PYRIDINE (M)
NH_4HCO_3 (M)
FRACTION NO.
0
4
8
12
16
20
24
0.2
1.0
2.0
0.1
0.2
0.3
0.4
12
24
36
48
60
72
86
96
0
6
12
18
24
30
36
42
48
54

Fig 8

30240-1

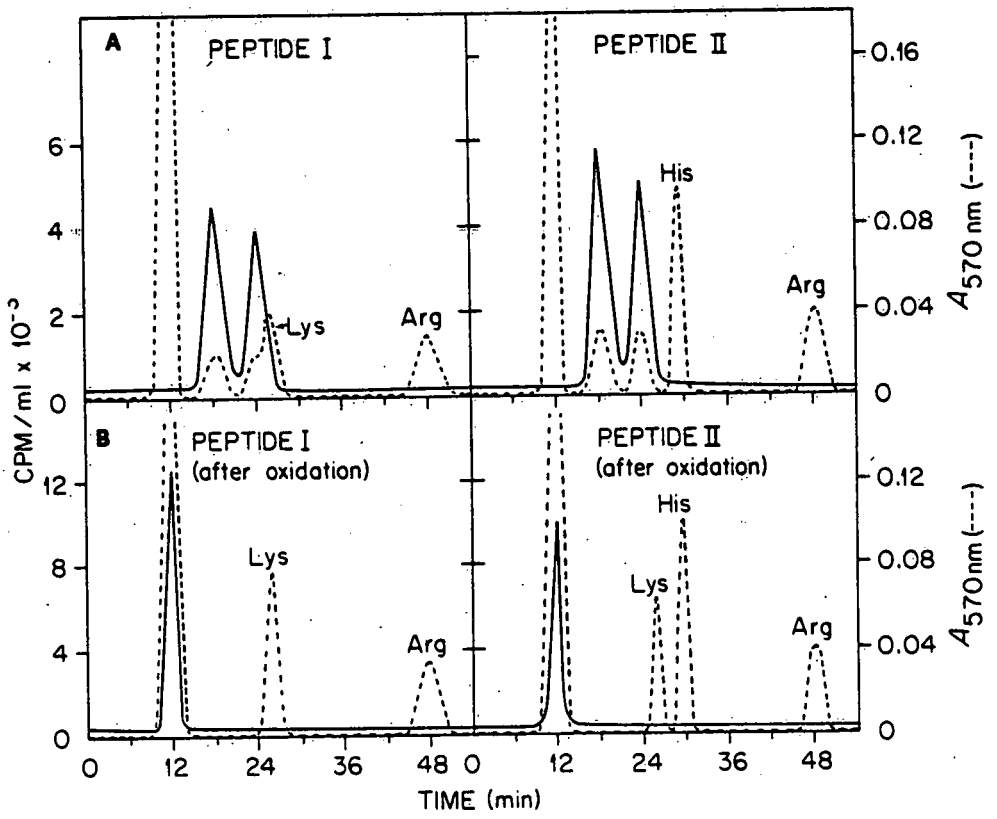
A
PEPTIDE I
PEPTIDE II
Lys
Arg
His
Arg
B
PEPTIDE I
(after oxidation)
PEPTIDE II
(after oxidation)
Lys
Arg
His
Lys
Arg
CPM/ml x 10⁻³
A570 nm (---)
A570nm (---)
TIME (min)
0
12
24
36
48
0.16
0.12
0.08
0.04

Fig. 9

34785

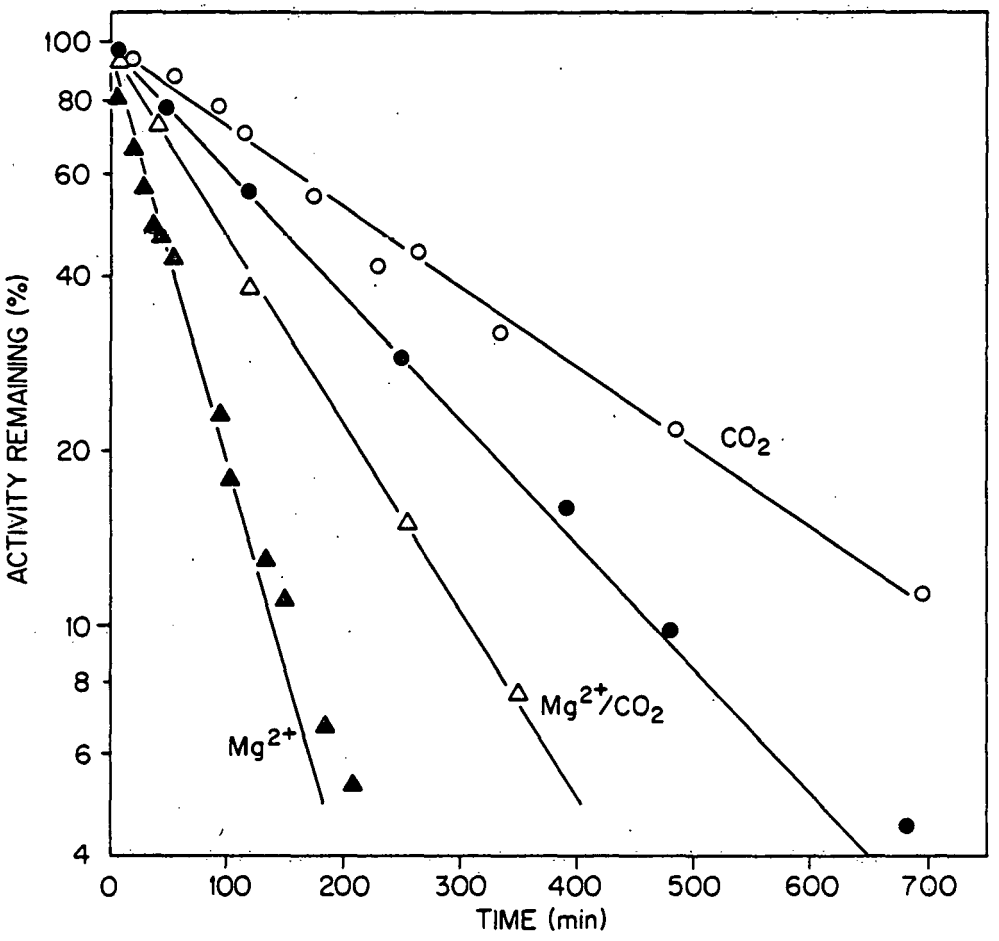
ACTIVITY REMAINING (%)
100
80
60
40
20
10
8
6
4
0
100
200
300
400
500
600
700
TIME (min)
CO_2
Mg^{2+}/CO_2
Mg^{2+}

Fig. 10

33187

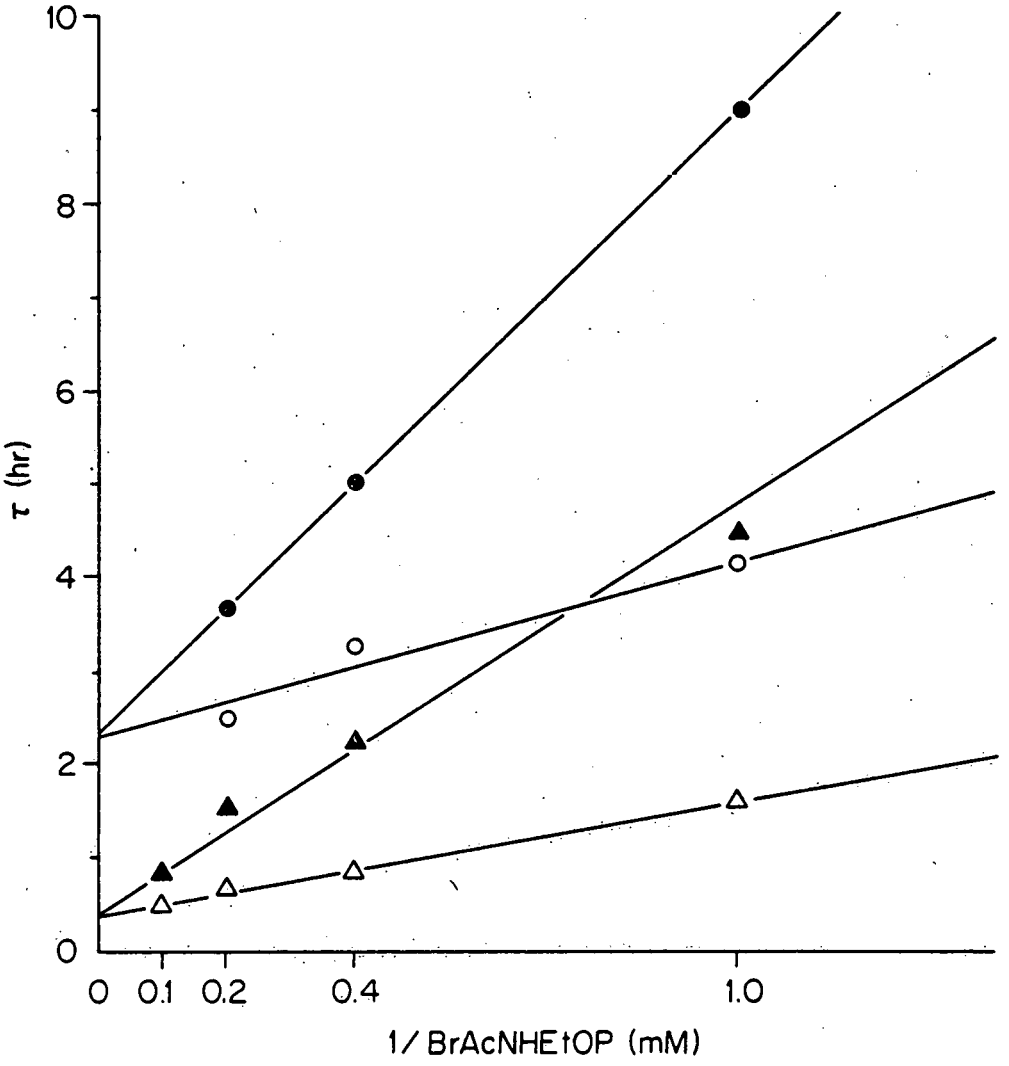

fig 11

3435-5

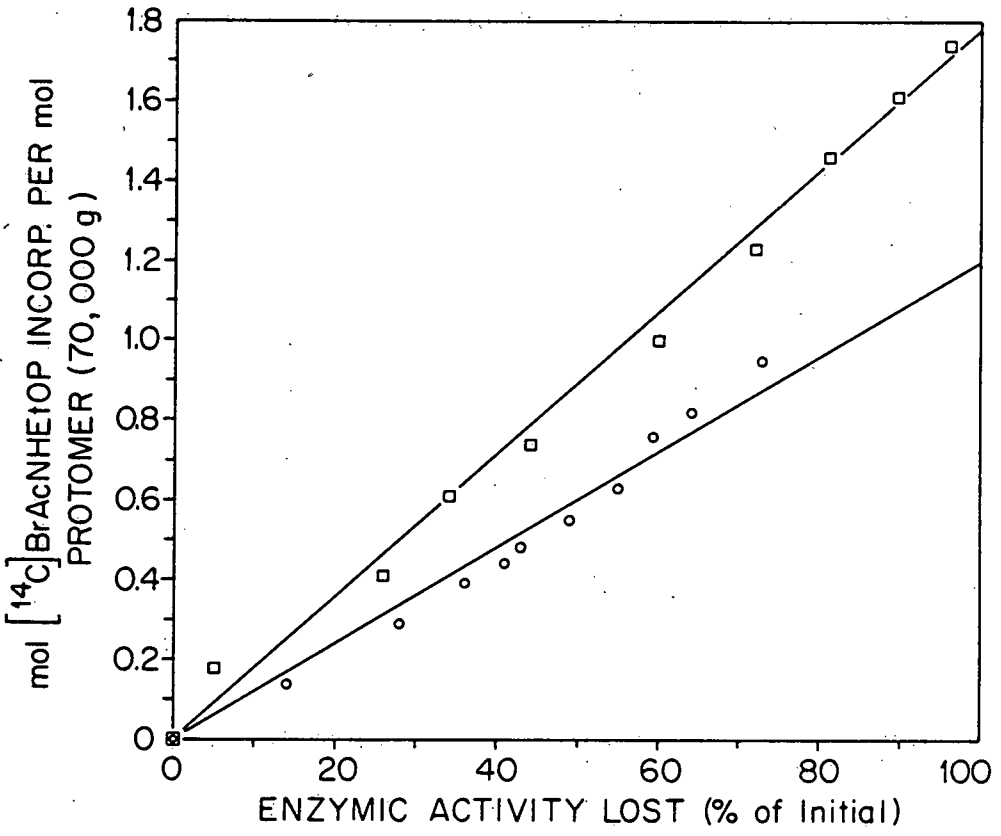
mol [14C]BrAcNHEtOP INCORP. PER mol PROTOMER (70,000 g)
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
0
20
40
60
80
100
ENZYMIC ACTIVITY LOST (% of Initial)

fig 12

33432-1

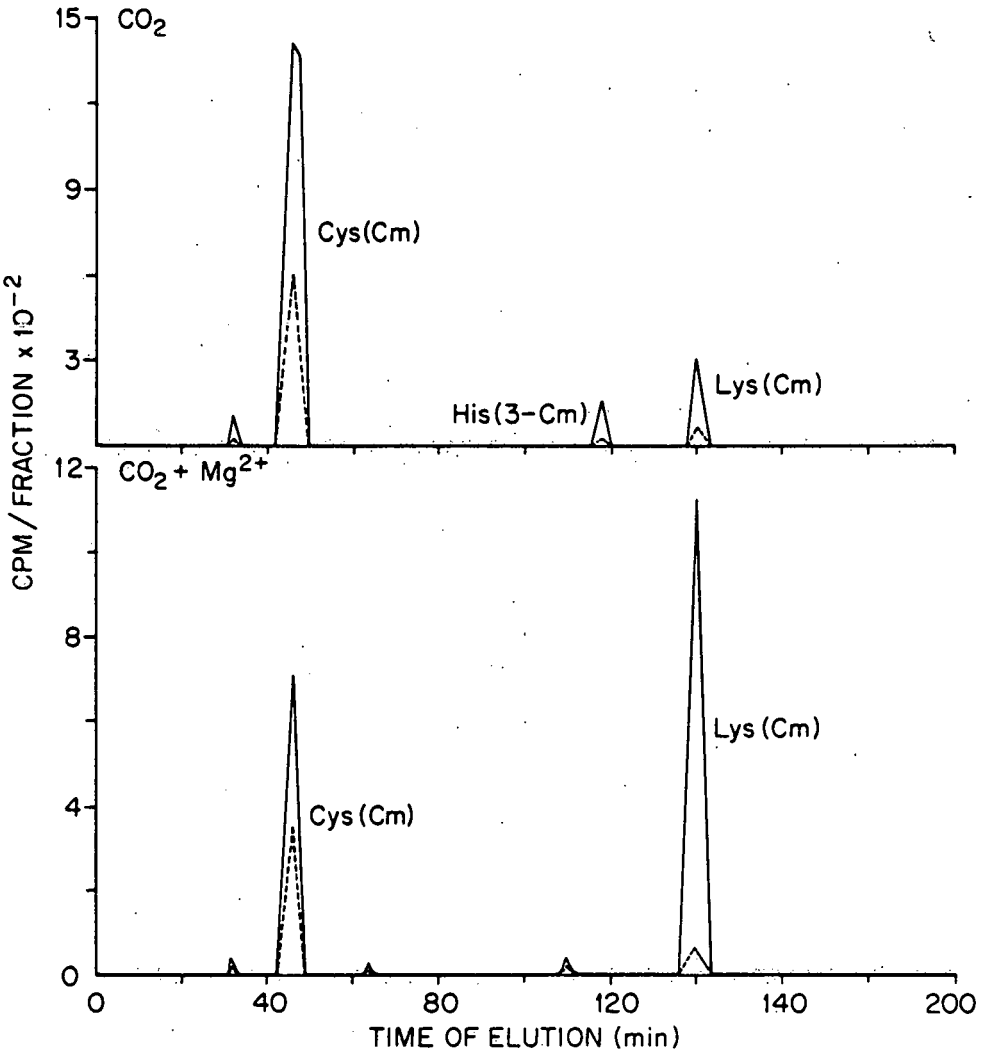

Fig. 13

34356

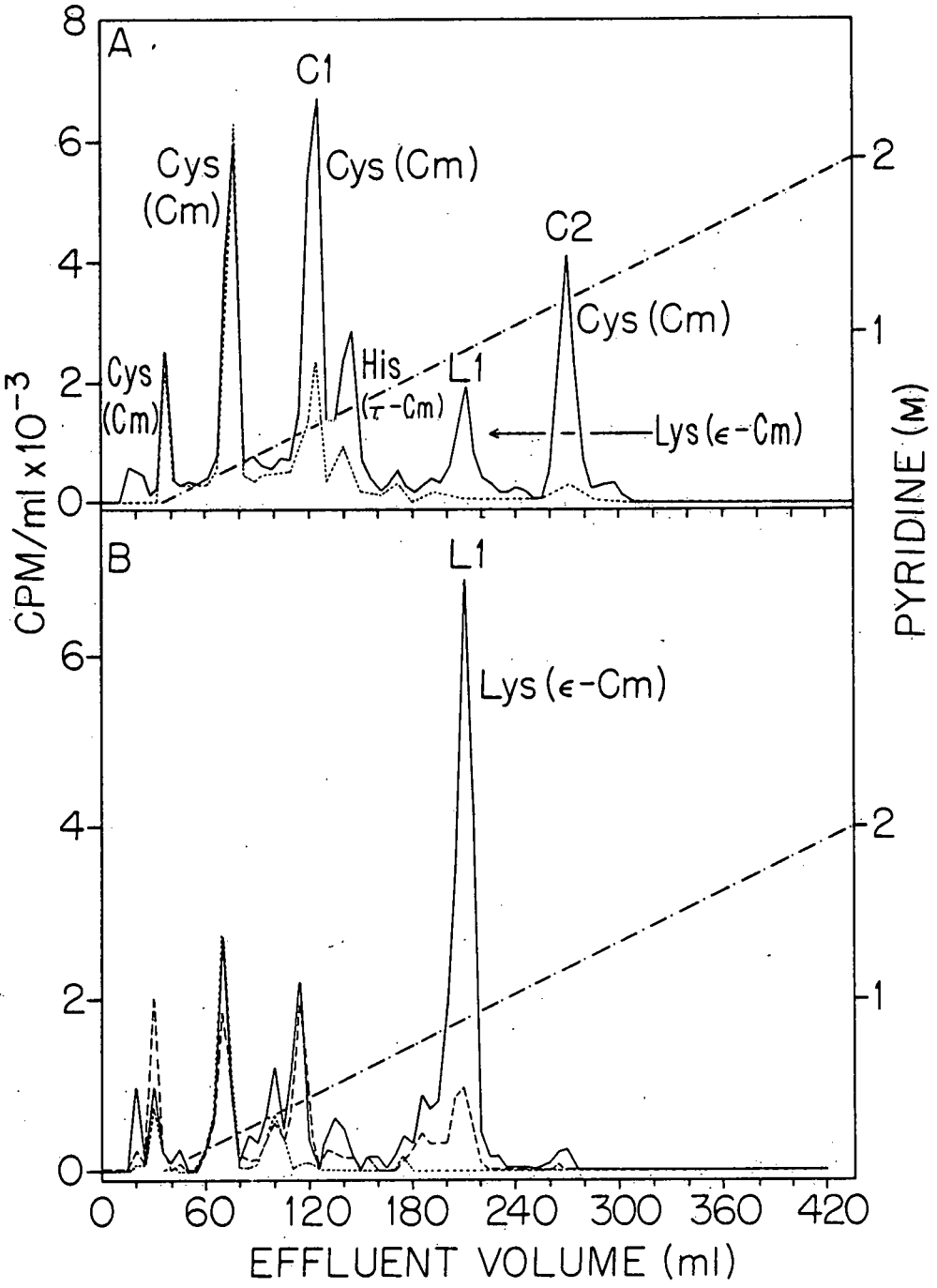
A
B
C1
C2
L1
L1
Cys (Cm)
Cys (Cm)
Cys (Cm)
Cys (Cm)
Cys (Cm)
His (τ-Cm)
Lys (ε-Cm)
Lys (ε-Cm)
CPM/ml x10⁻³
PYRIDINE (M)
EFFLUENT VOLUME (ml)
0
60
120
180
240
300
360
420
0
2
4
6
8
1
2

Fig 14