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The active site of Rubisco

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Previous affinity-labelling studies and comparative sequence analyses have identified two different lysine residues at the active site of ribulose bisphosphate carboxylase–oxygenase and have suggested that they are essential to function. The essential lysine residues occupy positions 166 and 329 in the *Rhodospirillum rubrum* enzyme and positions 175 and 334 in the spinach enzyme. Based on the pH-dependences of inactivations of the two enzymes by trinitrobenzene sulphonate, Lys 166 (*R. rubrum* enzyme) exhibits a pK_a of 7.9 and Lys 334 (spinach enzyme) exhibits a pK_a of 9.0. These low pK_a values, as well as the enhanced nucleophilicities of the lysine residues, argue that both are important to catalysis rather than to substrate binding. Lys 166 may correspond to the essential base that initiates catalysis and that displays a pK_a of 7.5 in the pH-curve for V_{max}/K_m. Cross-linking experiments with 4,4′-diisothiocyano-2,2′-disulphonate stilbene demonstrate that the two active-site lysine residues are within 12 Å of each other (1 Å = 10^{-10} m).

Introduction

One facet of acquiring a complete understanding of any enzyme mechanism is characterization of the active site. In the case of RuBP carboxylase (D-ribulose 1,5-bisphosphate carboxylase–oxygenase, EC 4.1.1.39), a consideration of its well-established reaction pathway reveals the minimal requirements for catalysis. As depicted in figure 1, the complex pathway entails enolization, carboxylation, hydration, carbon–carbon scission, and protonation (Miziorko & Lorimer 1983; Jaworowski & Rose 1985). Acid–base groups may facilitate or be required for: (*a*) polarization of the carbonyl group of RuBP; (*b*) abstraction of the C3 proton of RuBP; (*c*) stabilization of the C2 carbanion of RuBP; (*d*) polarization of CO_2 to enhance carboxylation of the enediolate intermediate; (*e*) hydration and concomitant carbon–carbon scission of the 2-carboxy-3-keto intermediate; (*f*) stabilization of the C2 carbanion of glycerate 3-phosphate, an especially important step because of the inversion of configuration required to form the D-isomer (Pierce *et al.* 1980); and (*g*) protonation of the carbanion of glycerate 3-phosphate.

Extensive NMR (Miziorko & Mildvan 1974; Pierce & Reddy 1986) and EPR studies (Miziorko & Sealy 1984; Styring & Brändén 1985) have provided strong evidence that the essential divalent cation, Mg^{2+}, is responsible for polarization of the carbonyl group and for stabilization of carbanion intermediates. Some of these studies became feasible only with the demonstration that a stable exchange-inert quaternary complex containing stoichiometric amounts of enzyme, CO_2, metal ion and 2-carboxyarabinitol bisphosphate (a transition-state analogue bearing a hydroxyl group instead of the carbonyl group as in the six-carbon reaction intermediate) was readily isolable by gel filtration (Miziorko & Sealy 1980). In essence, the EPR investigations utilizing the quaternary complex have established direct binding of metal ion to the carboxyl oxygens of 2-carboxyarabinitol bisphosphate and, by inference, to the corresponding oxygens in the six-carbon intermediate. The recent NMR experiments demonstrate that the metal ion

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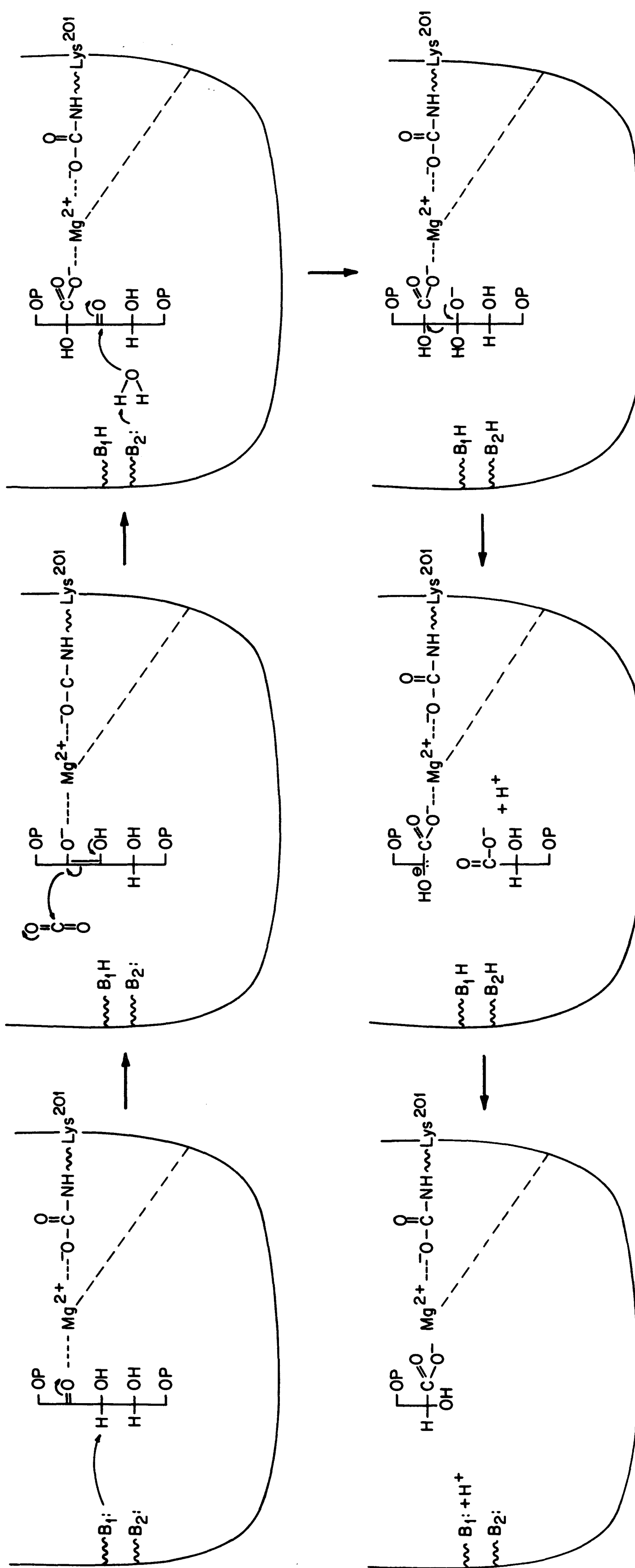

FIGURE 1. Reaction pathway for carboxylation of RuBP as catalysed by RuBP carboxylase (Miziorko & Lorimer 1983; Jaworowski & Rose 1985).

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is also close to the carbamate carbon, but not necessarily coordinated directly by the carbamate oxygens (Pierce & Reddy 1986). Protein ligands for the metal ion have been demonstrated but not identified (Miziorko *et al.* 1982).

Mg^{2+}-bound water, detected by EPR (Miziorko & Sealy 1984), has been invoked both as the essential base that abstracts the C3 proton of RuBP (Christeller 1982) and as the water molecule required for carbon–carbon scission of the carboxylated intermediate (Nilsson & Brändén 1983). If both of these suggestions are correct, a seemingly untenable situation arises, in which carboxylase activity is attributable to hydrated Mg^{2+} and the only role for the protein is as a template for binding and orienting metal ion and substrates. Because of the observed pH-dependences of V_{max}, K_m, and the deuterium isotope effect with [3-^{2}H] RuBP as substrate (Christeller 1982; Schloss 1983; Paech 1985), we infer the intervention of two protein acid–base groups ($-B_1$ and $-B_2$) in catalysis. From stereochemical considerations (Lorimer, this symposium), the base ($-B_1$) that abstracts the C3 proton to enolize RuBP could also promote hydration of the six-carbon intermediate but cannot represent the conjugate acid ($-B_2H$) that protonates the carbanion of glycerate 3-phosphate.

An unusual structural feature of the catalytic site is covalently bound CO_2. RuBP carboxylase requires CO_2 and Mg^{2+} for conversion of a deactivated form to a catalytically competent form. The activation process entails condensation of CO_2 with an ε-amino group, Lys 201 in the spinach enzyme (Lorimer 1981) and Lys 191 in the *Rhodospirillum rubrum* enzyme (Donnelly *et al.* 1983), to form a carbamate that is stabilized by Mg^{2+} (Miziorko 1979; Pierce *et al.* 1980; Hall *et al.* 1981). The identity of the lysine residue that reacts with CO_2 was provided by methylation of the carbamate in the isolated quaternary complex of enzyme–activator CO_2–Mg^{2+}–transition state analogue. Whether enzyme activation results from the conversion of a positively charged side-chain to a negatively charged one, the induction of a conformational change, or the introduction of a ligand for direct coordination of Mg^{2+}, is uncertain. Direct coordination is supported by the results of site-directed mutagenesis, in which replacement of the reactive lysine residue with a glutamate residue resulted in loss of the tight complexing of Mg^{2+} by the enzyme (Estelle *et al.* 1985).

RuBP could conceivably interact with a protein through condensation of its carbonyl with a cysteinyl sulphydryl or lysyl ε-amino group to form a thiohemiketal or a Schiff base, respectively. Although the former had been proposed on the basis of chemical modification of the carboxylase with sulphydryl reagents (Trown & Rabin 1964), both possibilities have been discounted by the retention of the C2-oxygen of RuBP during catalytic turnover (Lorimer 1978; Sue & Knowles 1978).

Given the competitive inhibition of RuBP carboxylase by a wide variety of phosphoesters and other anions, salt bridges between the anionic phosphate groups of RuBP and cationic groups of the enzyme probably represent the major mode of enzyme–substrate interaction (Paech *et al.* 1978). Because of the propensity of arginine residues to serve as anionic binding sites (Riordan *et al.* 1977), the reactions of the carboxylase with two different arginine reagents (phenylglyoxal and 2,3-butanedione) have been examined (Lawlis & McFadden 1978; Schloss *et al.* 1978*a*; Chollet 1981). Although the observed inactivation, substrate protection, and selectivity with phenylglyoxal are generally consistent with the presence of one or two active-site arginine residues that could be involved in substrate binding, definitive evidence is lacking. The lability of the arginine–reagent adduct has precluded establishing a direct correlation between inactivation and derivatization of a specific arginine residue. In contrast, a wealth of chemical

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data has revealed the presence of two different lysine residues (in addition to the one that forms a carbamate) at the active site of RuBP carboxylase, but both appear to be involved catalytically rather than in substrate binding. The possible functions of these two lysine residues and their spatial relationship are the major topics of the present paper.

Affinity labelling and comparative sequence studies

One focus of our laboratory has been the identification of active-site residues of RuBP carboxylase with chemical probes. Because of their inherent specificity and their potential for revealing mechanistic information, affinity labels are frequently superior to general protein reagents as a means of mapping active sites. Structurally, affinity labels resemble substrates but contain chemically reactive substituents that can covalently modify amino acid side-chains. The substrate-like features of the reagent direct it to the active site in a manner completely analogous to the binding of competitive inhibitors. This binding step results in a high localized concentration of reagent within the substrate binding site, increasing the likelihood of modification of a residue within this site as compared with other positions in the protein molecule.

The structures of five different affinity labels for RuBP carboxylase and their primary targets within the enzyme from both spinach and *R. rubrum* are shown in figure 2. Criteria for affinity labelling that are generally fulfilled by some or all of these reagents include the following:

(*a*) Complete inactivation as expected, provided that the residue subject to modification is essential to catalysis or substrate binding.

(*b*) Pseudo-first-order loss of activity at high molar ratios of reagent : enzyme, suggesting that inactivation correlates with the modification of one residue.

(*c*) Rate-saturation, indicative of reversible binding of the reagent to the active site as an obligatory step preceding inactivation. Such a pathway requires that the rate of inactivation is proportional to the concentration of enzyme–reagent complex; thus, as the reagent concentration is increased, the rate of inactivation approaches a limiting, finite value (Kitz & Wilson 1962; Meloche 1967).

(*d*) Protection by RuBP or competitive inhibitors against inactivation, with kinetics of protection that are consistent with reagent and protector competing for the same binding site.

(*e*) Competitive inhibition by the reagent over short time periods. Furthermore, the apparent binding constant of the given reagent based on competitive inhibition is the same as that calculated from the rate-saturation data for enzyme inactivation.

(*f*) A direct correlation between inactivation and modification of a particular residue as shown with radioactive reagents.

(*g*) A rate of inactivation much more rapid than the rate at which the reagent modifies the analogous type of amino acid residue in model peptides.

(*h*) Elimination of specificity of modification by disruption of tertiary structure with a protein denaturant, demonstrating the requirement for a functional substrate-binding site.

(*i*) Dramatic alterations in susceptibility of the enzyme to the reagents, or in the amino acid residues modified, when Mg^{2+} and CO_2 are excluded from the reaction mixture; i.e. the deactivated and activated forms of the carboxylase exhibit different reactivities toward site-specific reagents.

The structural complexity of RuBP carboxylase and the lack of absolute specificity of the

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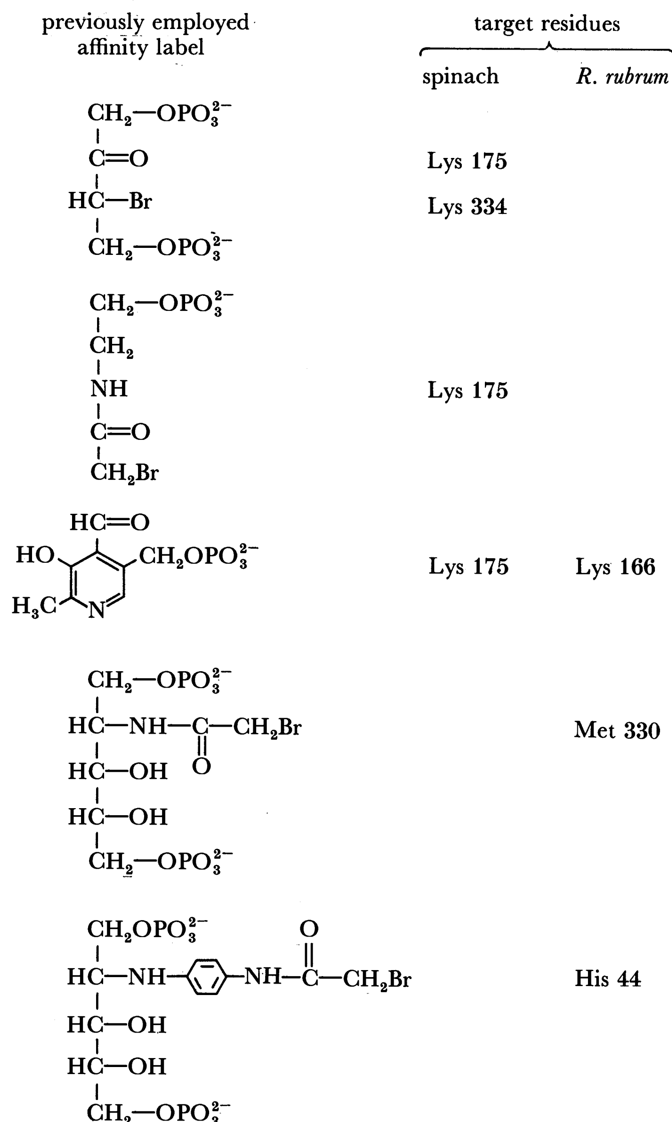

FIGURE 2. Affinity labels for RuBP carboxylase and the targets of modification. From top to bottom, the reagents are 3-bromo-1,4-dihydroxy-2-butanone 1,4-bisphosphate, *N*-bromoacetylethanolamine phosphate, pyridoxal phosphate, 2-bromoacetylaminopentitol 1,5-bisphosphate, and 2-(4-bromoacetamido)anilino-2-deoxypentitol 1,5-bisphosphate.

affinity labels used have prompted us to rely on comparative sequence analyses to reveal whether residues implicated at the active site by affinity labelling are indeed species-invariant and thus likely to be essential to function (Hartman *et al.* 1984; Nargang *et al.* 1984). Because of their evolutionary diversity and structural dissimilarities, the carboxylases from spinach and *R. rubrum* provide a stringent test for structural conservation. Whereas the quaternary structure of RuBP carboxylase from spinach is typical of that observed among all higher plant and most bacterial carboxylases in that it is a hexadecamer with eight large (53 kDa) and eight small (14 kDa) subunits (Rutner 1970; Martin 1979; Zurawski *et al.* 1981), the functionally analogous enzyme from the purple, non-sulphur photosynthetic bacterium *R. rubrum* is a homodimer of 53 kDa subunits (Tabita & McFadden 1974; Schloss *et al.* 1982; Hartman *et al.* 1984).

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Futhermore, despite greater than 80% sequence homology among most RuBP carboxylases (Miziorko & Lorimer 1983), the homology between the *R. rubrum* enzyme and the large subunit of the spinach carboxylase is only 31% (Hartman *et al.* 1984; Nargang *et al.* 1984).

Studies with the affinity labels shown in figure 2 have provided evidence that at least three regions of the polypeptide compose the catalytic site (see Hartman *et al.* 1984 for a review). In each case, the residues, for which sound chemical evidence has suggested functional importance, are located within highly conserved regions among diverse species (figure 3). Credence is thus lent to the concept of universality of mechanism of RuBP carboxylases and their evolution from a single ancestral gene.

FIGURE 3. Amino acid sequences of RuBP carboxylase from spinach (upper) (Zurawski *et al.* 1981) and *R. rubrum* (lower) (Hartman *et al.* 1984; Nargang *et al.* 1984). Alignments were made by visual inspection. Residues identical in both enzymes are enclosed in boxes. Gaps, attributed to deletions or insertions, appear as blank spaces. Peptide notations are explained in Hartman *et al.* 1984. Cysteine residues and residues identified at the active site or activator site by selective chemical labelling are illustrated with larger type. Abbreviations: Asp, D; Asn, N; Glu, E; Gln, Q; Thr, T; Ser, S; Pro, P; Gly, G; Ala, A; Cys, C; Val, V; Met, M; Ile, I; Leu, L; Tyr, Y; Phe, F; His, H; Lys, K; Trp, W; Arg, R. (Reprinted from Hartman *et al.* (1984) with permission of the publisher.)

Lys 175 in the spinach enzyme is selectively labelled by three different reagents: 3-bromo-1,4-dihydroxy-2-butanone 1,4-bisphosphate, *N*-bromoacetylethanolamine phosphate and pyridoxal 5′-phosphate. The corresponding residue in the *R. rubrum* enzyme, Lys 166, is modified by pyridoxal phosphate with a high degree of specificity. An identical seven-residue sequence

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encompasses this reactive lysine residue in the two species. A second active-site region contains Lys 334 in spinach carboxylase and Met 330 in the *R. rubrum* enzyme. The former is labelled by 3-bromo-1,4-dihydroxy-2-butanone 1,4-bisphosphate and the latter by 2-bromoacetylaminopentitol 1,5-bisphosphate. Although Met 330 is replaced by Leu 335 in the spinach enzyme and hence cannot function in catalysis, Lys 334 and eight other residues in its immediate vicinity are conserved. Thus two different lysine residues (Lys 175 and Lys 334 in the spinach enzyme, which correspond to Lys 166 and Lys 329 in the *R. rubrum* enzyme) appear essential. A region encompassing His 44 in *R. rubrum* carboxylase is implicated by the selective labelling of this residue with 2-(4-bromoacetamido)anilino-2-deoxypentitol 1,5-bisphosphate. The residue that is subject to selective alkylation by the affinity label apparently does not function in catalysis, because it is deleted in the enzyme from spinach. However, 11 of the 14 residues from positions 40 to 54 are conserved; this result is strongly suggestive of functional participation of at least one of the invariant residues. High homology is also observed around Lys 201, the residue that reacts with CO_2, resulting in activation of the enzyme (labelled 'activator-site peptide' in figure 3).

Acidities of two active-site lysine residues

Although the affinity-labelling studies, combined with comparative sequence analyses, have provided rather compelling evidence that RuBP carboxylase contains two essential lysine residues, besides the regulatory one that forms a carbamate, their precise functions are not known. To place limitations on what their roles might be, we have sought to determine the pK_a values of the essential lysine residues (Lys 166 and Lys 329 in the *R. rubrum* enzyme and Lys 175 and Lys 334 in the spinach enzyme).

In principle, the pH-dependence of inactivation by the affinity labels should provide the pK_a values of the targeted residues; however, this approach failed because of the pH-dependences of the reagents' binding affinities due to ionization of the phosphate groups over the pH-range of interest. Another difficulty, irrespective of reagent used, is that the CO_2–Mg^{2+}-induced activation is pH-dependent (Lorimer *et al.* 1976); this dependence necessitates focusing on a pH-range over which the enzyme can be maintained in its fully activated state. To circumvent the problem imposed by changes in ionization state of previously designed affinity labels, we have screened lysine-selective reagents for their ability to modify preferentially the active-site lysine residues and have discovered that 2,4,6-trinitrobenzenesulphonate (TNBS) selectively arylates Lys 166 in the *R. rubrum* enzyme and Lys 334 in the spinach enzyme (Hartman *et al.* 1985).

In the presence of CO_2 and Mg^{2+}, the fully activated forms of both the *R. rubrum* and spinach enzymes are rapidly inactivated by TNBS in pseudo-first-order fashion. Saturating levels of carboxyribitol bisphosphate (0.1 mM), a competitive inhibitor with $K_i = 2\mu M$ (Pierce *et al.* 1980; Schloss *et al.* 1982), afford only partial protection. Since the rates of inactivation are directly proportional to the TNBS concentration (up to 2 mM), there is no indication that the enzymes have an affinity for TNBS.

To determine the pK_a value of any amino acid residue within an enzyme from the pH-dependence of its inactivation by a chemical reagent, a precise correlation between site of modification and loss of catalytic activity must be established. This criterion is most easily met if the modification is specific, i.e. is restricted to a single amino acid side-chain. TNBS fulfils

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this requirement in its reaction with RuBP carboxylase as determined by characterization of tryptic digests of the inactivated enzyme.

The enzymes from *R. rubrum* and spinach were treated with TNBS under both activation and deactivation conditions (50 mM Bicine, pH 8.0, with or without 10 mM $MgCl_2$/66 mM $NaHCO_3$); the inactivated samples were then carboxymethylated and digested with trypsin. Unfractionated tryptic digests of the four samples were inspected by reverse-phase HPLC monitored at either 215 nm for detection of all peptides or 367 nm for detection of TNP-peptides. Figure 4*a* shows that only one TNP-peptide of significance is present in digests of the inactivated spinach carboxylase, irrespective of whether the enzyme was modified under activation or deactivation conditions. Although the digest of the *R. rubrum* enzyme that was treated with TNBS under activation conditions also contains only one major TNP-peptide, the corresponding

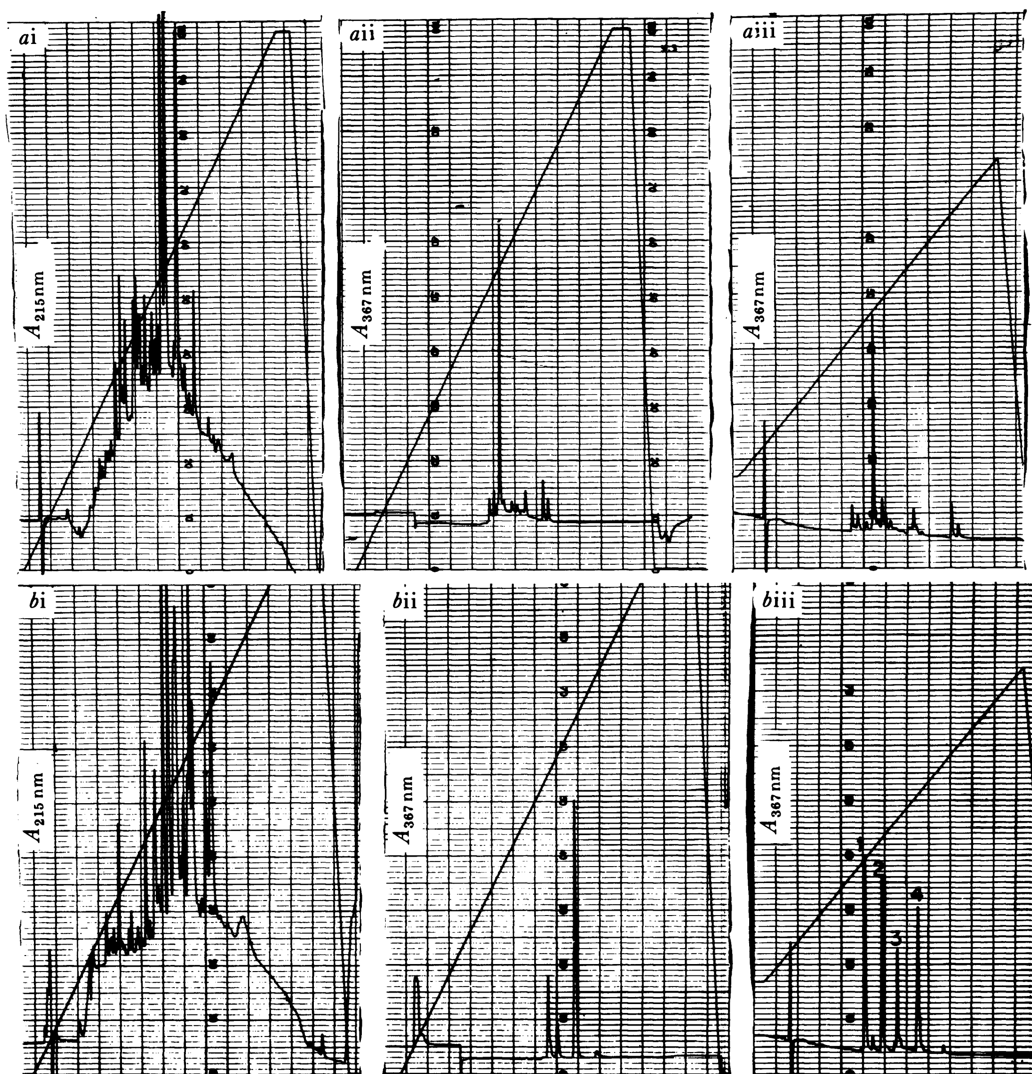

FIGURE 4. HPLC profiles of tryptic digests of spinach RuBP carboxylase (*a*) and *R. rubrum* RuBP carboxylase (*b*) that had been treated with TNBS. Panels *a*i and *b*i show all peptides detected at 215 nm; the other four panels show only TNP-peptides detected at 367 nm. Panels *a*ii and *b*ii represent samples of enzymes (duplicates of those shown in panels *a*i and *b*i, respectively) inactivated by TNBS under activation conditions; panels *a*iii and *b*iii represent samples of enzymes inactivated by TNBS under deactivation conditions. Note that the gradient is not the same in every panel, and thus elution positions cannot be compared directly.

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digest of TNBS-treated deactivated enzyme contains four major labelled peptides (figure 4*b*). The TNP-peptides from digests of both enzymes were purified by diethylaminoethyl (DEAE)-cellulose chromatography, gel filtration and HPLC, in succession. Amino acid analyses of these peptides permit the following assignments of their locations within the respective polypeptide chains (see figure 3):

(*a*) Peptide from spinach enzyme (figure 4*a*ii and 4*a*iii): fragment 320–339 with TNP-Lys at position 334.

(*b*) Peptide from *R. rubrum* enzyme (modified under activation conditions, figure 4*b*ii): fragment 149–168 with TNP-Lys at position 166.

(*c*) Peptides from *R. rubrum* enzymes (modified under deactivation conditions, figure 4*b*iii):

peptide 1: fragment 289–301 with TNP-Lys at position 300.
peptide 2: fragment 1–6 with *N*-α-TNP-Met at position 1.
peptide 3: fragment 314–337 with TNP-Lys at position 329.
peptide 4: fragment 149–168 with TNP-Lys at position 166.

To satisfy a frequently overlooked criterion for using the pH-dependence of chemical modification to determine the pK_a of a residue, we demonstrated an unaltered reagent specificity throughout the pH-range examined. In the cases of the arylations reported herein, the tryptic patterns presented in figure 4 were not significantly changed when the enzymes were derivatized at either pH 6.5 or pH 9.5, the extremes of pH used for modifications.

A third condition that must be met with the carboxylase, due to its pH-dependent activation by CO_2–Mg^{2+}, is verification that changes in its activation state do not occur over the pH-range of interest. The activation of RuBP carboxylase by CO_2–Mg^{2+} entails conformational changes, which can be detected by physical methods (Siegel & Lane 1972; Miziorko & Sealy 1980) and are reflected in altered ligand-binding properties (Jordan & Chollet 1983; Jordan *et al.* 1983) and altered accessibility of some amino-acid residues to chemical reagents (Schloss *et al.* 1978*b*; Hartman *et al.* 1984). By diluting fully-activated stocks of the enzyme (in pH 8.0 buffer containing saturating levels of CO_2 and Mg^{2+}) into buffers of various pH (also containing saturating concentrations of CO_2 and Mg^{2+}), the deactivation process could be monitored with the $^{14}CO_2$-fixation assay. This assay readily distinguishes activated from deactivated carboxylase, because the time required to measure the enzyme activity (30 s) is short compared to the rate of activation, which is greatly slowed by RuBP (Lorimer *et al.* 1977; Jordon & Chollet 1983). The deactivated enzyme exhibits less than 10% of the activity of the fully activated enzyme in this assay (Lorimer *et al.* 1977). These experiments demonstrated that, from pH 6.5 to pH 9.5, the fully activated state is maintained for at least two hours.

The reaction of TNBS with amines is second-order, and for most compounds examined the pH-dependences show that only unprotonated amino groups are reactive (Freedman & Radda 1968; Goldfarb 1966). The observed second-order rate constant (k_2) will thus reflect the degree of ionization so that the ionization constant (K_a) can be readily calculated. Figure 5 illustrates the pH-dependences of the reactions of TNBS with *N*-α-acetyllysine, activated *R. rubrum* carboxylase, activated spinach carboxylase and deactivated spinach carboxylase. The data are plotted in two different ways: log k_2 against pH for the entire pH-range examined, and $1/k_2$ against [H^+] over an appropriate narrow pH range (insets). The calculated pK_a values and intrinsic reactivities (k_0) for acetyllysine, Lys 166 of the activated *R. rubrum* enzyme, and Lys 334 of the spinach enzyme (under both activation and deactivation conditions) are provided in table 1. Particularly noteworthy are the striking nucleophilicities (k_0) of the two protein ε-amino

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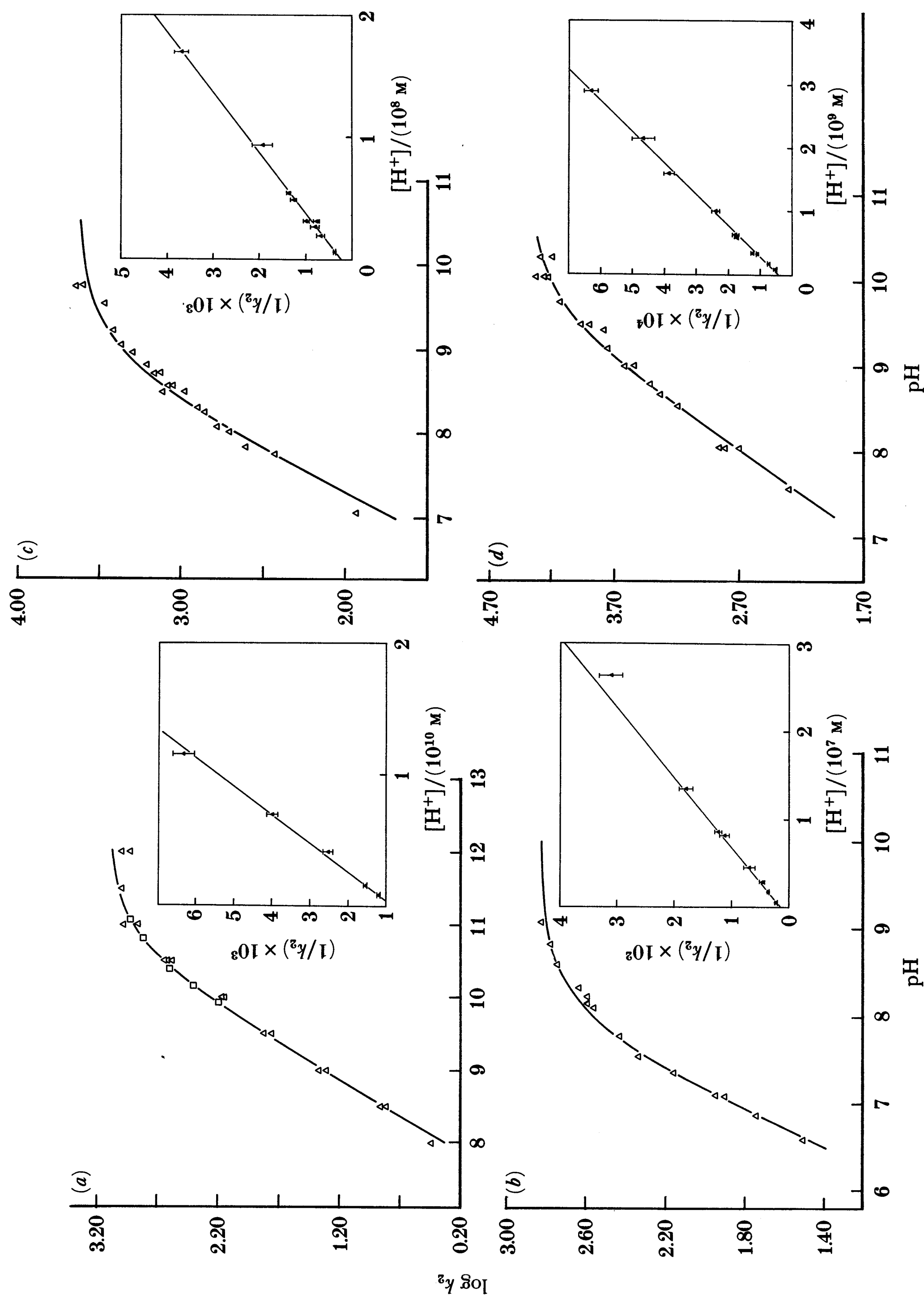

FIGURE 5. pH-dependences of the reactions of TNBS with *N*-α-acetyllysine (*a*), activated *R. rubrum* RuBP carboxylase (*b*), activated spinach RuBP carboxylase (*c*), and deactivated spinach RuBP carboxylase (*d*).

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TABLE 1. OBSERVED SECOND-ORDER RATE CONSTANTS FOR THE REACTIONS OF TNBS WITH *N*-α-ACETYLLYSINE AND WITH RuBP CARBOXYLASE

sample	pK_a	k_0 / $M^{-1}\,min^{-1}$	k_2 / $M^{-1}\,min^{-1}$	
			pH 7.0	pH 8.0
N-α-acetyllysine	10.8	1250	0.2	2.0
Lys 166 of activated *R. rubrum* enzyme	7.9	670 (0.54)†	73 (365)	364 (182)
Lys 334 of activated spinach enzyme	9.0	4500 (3.6)	46 (230)	409 (205)
Lys 334 of deactivated spinach enzyme	9.8	26000 (20.8)	41 (205)	406 (203)

† Values in parentheses are relative to those of acetyllysine.

groups compared to acetyllysine, despite their stronger acidities. Curiously, the differences in k_0 and pK_a for Lys 166 and Lys 334 counterbalance such that the observed reactivities (k_2) at pH 7.0 and pH 8.0 are very similar.

Because the site-specificity of TNBS for the spinach enzyme is insensitive to its activation state, an opportunity is provided to ascertain whether the ionization constant for active-site Lys 334 of the spinach enzyme is altered as a consequence of the conformational changes associated with the activation and deactivation process. The present study clearly demonstrates that, in the activated enzyme, as compared to the deactivated counterpart, Lys 334 is considerably more acidic (pK_a of 9.0 vs. 9.8) and less reactive (k_0 of 4500 vs. 26000 $M^{-1}\,min^{-1}$). During activation, movement of the Lys 334 side chain into a more hydrophobic environment with less accessibility to solvent could explain the increased acidity.

Previous correlation between basicity of amino acids (and small peptides) and reactivity towards TNBS predicts that amino groups with pK_a values of 7.9 (Lys 166 of the *R. rubrum* enzyme), 9.0 (Lys 334 of the activated spinach enzyme), and 9.8 (Lys 334 of the deactivated spinach enzyme) will have k_0 values of 120, 280, and 600 $M^{-1}\,min^{-1}$, respectively (Fields 1971). In contrast, the k_0 values determined experimentally are 670, 4500, and 26000 $M^{-1}\,min^{-1}$. These two active-site lysine residues are thus considerably more nucleophilic than anticipated from their pK_a values, and their selective arylation by TNBS is favoured by both enhanced acidity and enhanced nucleophilicity. This unusual situation might reflect the microenvironment at the catalytic site. An alternative explanation could be provided by selective reversible binding and orientation of TNBS so as to increase the rates of arylation. However, any such affinity must be quite weak based on the linear relationship between rate of inactivation and reagent concentration (i.e. lack of rate-saturation).

Although the unusual acidity and nucleophilicity can account for the specificity of TNBS for Lys 166 and Lys 334, the similar reactivities at pH 8.0 observed for the two residues (see table 1) raise the question as to why only one of them is modified in each species of carboxylase investigated. The most obvious explanation for the failure of both to react is that the two lysine residues are juxtaposed within the catalytic site so that derivatization of both is precluded on steric grounds. This argument was presented earlier to explain the mutual exclusiveness of the two active-site lysine residues in their reactivity toward 3-bromo-1,4-dihydroxy-2-butanone 1,4-bisphosphate (Norton *et al.* 1975). A weak non-covalent interaction between TNBS and the enzyme, albeit undetected by the experiments carried out, in which the reagent is rather

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precisely oriented, could explain the restriction of arylation to only one of the two potentially reactive lysine residues. The species-dependence of the lysine residue targeted would then be a consequence of rather subtle differences in active-site geometries. Interestingly, both Lys 329 and Lys 166 in the *R. rubrum* enzyme are arylated by TNBS under deactivation conditions (figure 4*b*iii), an observation which could be interpreted to indicate that the two residues are farther apart in the deactivated enzyme.

In addressing the question of a role in substrate binding versus a role in catalysis for the two active-site lysine residues, several observations argue against the former. The enhanced nucleophilicities of both lysine residues are more consistent with a catalytic involvement than with a function in binding. The incomplete protection of both enzymes against TNBS-inactivation by saturating levels of carboxyribitol bisphosphate is inconsistent with those lysine residues which are targets for arylation, forming salt linkages with phosphate groups of RuBP. The inactivated spinach carboxylase, in which Lys 334 has been arylated, is still able to form the quaternary complex with CO_2, Mg^{2+}, and carboxyarabinitol bisphosphate. With respect to Lys 166 in the *R. rubrum* carboxylase, its extreme acidity ($pK_a = 7.9$) appears incompatible with effective utilization as a phosphate-binding site. Finally, replacement of Lys 166 in the *R. rubrum* carboxylase with arginine by site-directed mutagenesis gives a gene product devoid of carboxylase activity (Larimer *et al.* 1985). If a protonated lysine residue were involved only in substrate binding, arginine substitution would be anticipated to yield a functional enzyme.

Catalytic roles that could be served by protonated lysine residues of RuBP carboxylase are polarization of CO_2 to facilitate its addition to the enediolate intermediate or protonation of the C2 carbanion of glycerate 3-phosphate (see $-B_2H$ in figure 1). With a pK_a of 9.0 (established by the TNBS studies), Lys 334 may be the essential acid with an apparent pK_a of 8.4 revealed by the pH-dependence of V_{max}/K_m (Schloss 1983).

Steps possibly subject to mediation by unprotonated lysine residues are abstraction of the C3 proton of RuBP to initiate enolization or hydration of the 2-carboxy-3-keto-intermediate (see $-B_1$ and $-B_2$, respectively, in figure 1). Based on the primary deuterium isotope effect with [3-^{2}H]RuBP, the proton-abstraction step is partly rate-limiting and thus contributes to the pH-profile of enzyme activity (Schloss 1983). A careful examination of the pH-dependences of both V_{max}/K_m and the deuterium isotope effect with the *R. rubrum* enzyme reveals that this essential base (as the conjugate acid) has a pK_a of 7.5 (J. V. Schloss, personal communication). Furthermore, the pK_a is not sensitive to the dielectric constant of the solvent; this suggests a cationic acid, i.e. a lysine or histidine residue. Thus, it is very tempting to equate Lys 166 ($pK_a = 7.9$) with the catalytic base that promotes enolization of RuBP.

The same function of proton abstraction has been assigned to His 298 in the spinach enzyme (or His 291 in the *R. rubrum* enzyme) on the basis of the pH-dependence of inactivation by diethyl pyrocarbonate (Paech 1985). Inactivation of the enzyme does correlate with modification of His 298 (Igarashi *et al.* 1985), but the lack of specificity of diethyl pyrocarbonate complicates the assignment of an inflection in the pH-curve to the ionization of a specific residue.

Distance between two active-site lysine residues

Because of the mutual exclusiveness of Lys 166 and Lys 329 of RuBP carboxylase in their reactions with various reagents (see preceding section), we speculated that these two active-site lysine residues must be in proximity. To challenge this postulate, we have explored the

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reactions of the *R. rubrum* enzyme with several chemical cross-linking agents. In each case, criteria prompting in-depth studies were inactivation by the reagent, protection by the competitive inhibitor carboxyribitol bisphosphate, and a reasonable degree of selectivity as judged by peptide mapping. One reagent that satisfies these criteria is 4,4′-diisothiocyano-2,2′-disulphonate stilbene (DIDS), which has been used as an irreversible anion-transport inhibitor (Cabantchik & Rothstein 1972). We were encouraged to try this reagent, which spans 12 Å†, because specificity for the active site seems to be enhanced by negatively charged, hydrophobic moieties. To relate 12 Å to active-site geometry, consider that if RuBP were bound in its fully extended conformation the distance between the two phosphorus atoms would be 11 Å; also, the experimentally measured distance between the essential divalent metal-ion and the phosphorus atoms of the substrate is within 6 Å (Pierce & Reddy 1986).

R. rubrum RuBP carboxylase is a dimer so that any cross-links introduced could be intrasubunit, intersubunit, or intermolecular. As only intrasubunit cross-links are pertinent to distances between residues within the active site, the DIDS-inactivated enzyme, after carboxymethylation of sulphydryls, was subjected to gel filtration in the presence of urea, thereby permitting the isolation of the monomeric polypeptide (figure 6). Samples of the

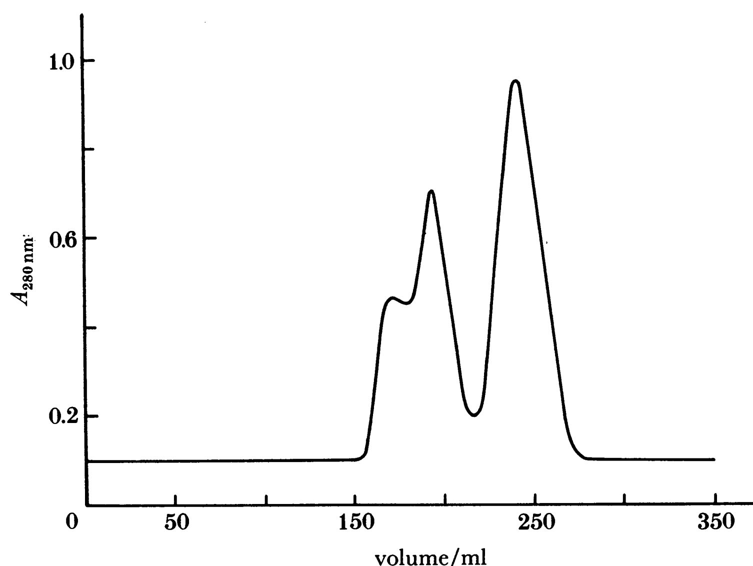

FIGURE 6. Gel filtration of DIDS-inactivated, carboxymethylated *R. rubrum* RuBP carboxylase on a 100 × 2.5 cm column of Ultragel AcA 34 (LKB). The column was equilibrated and eluted with 0.05 M Hepes (pH 6.8) containing 6 M urea and 10 mM dithiothreitol.

unfractionated inactivated enzyme and of the isolated monomeric fraction were digested with trypsin; these digests were inspected by HPLC to ascertain the degree of reagent specificity (figure 7). Based on the A_{342nm} (the major visible absorbance band of DIDS), over half of the incorporated reagent was associated with a single peptide. This peptide was purified by preparative ion-exchange chromatography on DEAE-cellulose, followed by gel filtration on Sephadex G-25.

The amino acid composition and sequence of the purified peptide demonstrated that it

† 1 Å = 10^{-10} m = 0.1 nm.

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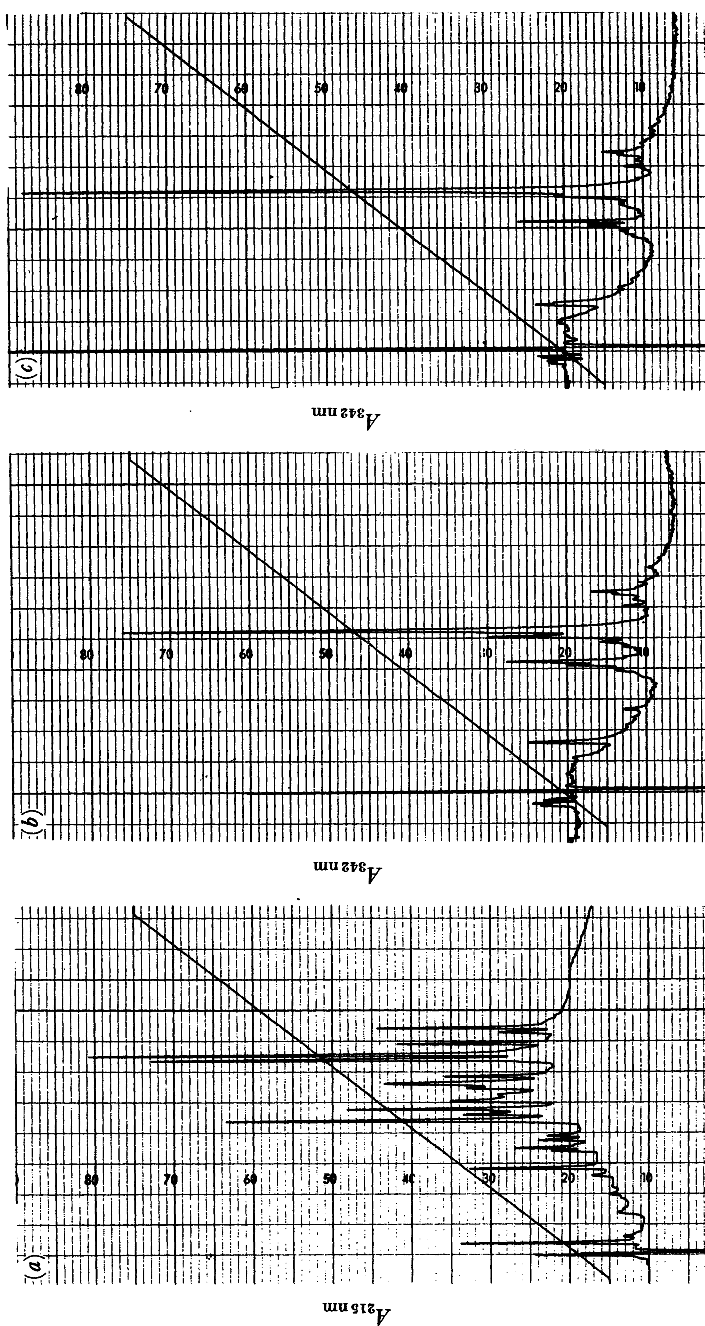

FIGURE 7. HPLC profiles of tryptic digests of DIDS-inactivated, carboxymethylated RuBP carboxylase: (*a*) shows all peptides detected at 215 nm; (*b*) and (*c*) show only modified peptides detected at 342 nm. The digest displayed in (*b*) is that of the unfractionated inactivated enzyme; the digest displayed in (*c*) is that of the monomeric fraction isolated by gel filtration (the component that elutes last, as illustrated in figure 6).

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consists of two chains encompassing positions 149–168 and 314–337 of the original protein subunit (see figure 3):

```
             150                              160
       NH2–V–L–G–R–P–E–V–D–G–G–L–V–V–G–T–I–I–K–P–K–COOH
                                                /
                        320                    /330
NH2–L–Q–G–A–S–G–I–H–T–G–T–M–G–F–G–K–M–E–G–E–S–S–D–R–COOH
```

The location of the covalent cross-link between Lys 166 and Lys 329 was revealed by the complete absence of phenylthiohydantoin-lysine at their respective positions during Edman degradation.

Although these experiments do not establish the precise distance between Lys 166 and Lys 329 during catalytic turnover, they do show that within the fully activated conformer the two active-site lysine residues can be juxtaposed only 12 Å apart.

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