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Further Characterization of an Essential Histidine Residue of Ribulose-1,5-bisphosphate Carboxylase/Oxygenase†

Christian Paech

Department of Chemistry, Station Biochemistry Section, South Dakota State University, Brookings, South Dakota 57007-1217

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ABSTRACT: Ribulose-1,5-bisphosphate carboxylase/oxygenase from spinach leaves was previously shown to have one essential histidine residue per active site [Bhagwat, A. S., & Ramakrishna, J. (1981) *Biochim. Biophys. Acta 662*, 181-189; Saluja, A. K., & McFadden, B. A. (1982) *Biochemistry 21*, 89-95]. Here, the pH dependence of the reactivity of the enzyme toward diethyl pyrocarbonate was used to delineate the pK_a value of this putatively essential histidine residue. A value of 6.85 at 15 °C and ionic strength I = 0.10 was experimentally obtained. Corrected for temperature and ionic strength, the pK_a became 6.54, in excellent agreement with a pK_a of 6.55 identified in a pH profile of V_{max}/K_m for ribulose 1,5-bisphosphate at 30 °C and ionic strength I = 0.14. These findings further substantiate the hypothesis that a histidine residue is involved in the catalytic mechanism of ribulose-1,5-bisphosphate carboxylase/oxygenase.

Lack of a detailed three-dimensional structure of ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBP carboxylase)[1] makes the kinetic analysis of chemical modificatin reactions still a viable option for investigating amino acid residues functional in the catalysis of carboxylation and oxygenation of RuBP. While RuBP carboxylase activity was shown to be sensitive to derivatization of lysine (Whitman & Tabita, 1976; Paech et al., 1977; Schloss et al., 1978b), arginine (Lawlis & McFadden, 1978; Schloss et al., 1978a; Chollet, 1981), histidine (Bhagwat & Ramakrishna, 1981; Saluja & McFadden, 1980, 198), tyrosine [Robison & Tabita, 1979; Bhagwat, 1982; but cf. Barnard et al. (1983)], cysteine (Rabin & Trown, 1964), and carboxyl (Valle & Vallejos, 1984) residues, only lysine-201 of the spinach enzyme was assigned a function in the mechanism of RuBP carboxylase by virtue of the fact that CO_2, as a homotropic effector, covalently binds to the ϵ-amino group of this lysine (Lorimer, 1981). To suggest the "essentiality" of other amino acid residues, one must use circumstantial evidence until such time that X-ray crystallography or other physical methods establish their presumed positions.

In the studies reported herein, we have used pH-dependent rates of ethoxyformylation of a single histidine residue of RuBP carboxylase (Bhagwat & Ramakrishna, 1981; Saluja & McFadden, 1982) to delineate its pK_a. Responses of K_m and V_{max} for RuBP to pH changes further support the notion that this histidine is involved in the catalysis of RuBP carboxylation.

Experimental Procedures

Materials

RuBP carboxylase was purified to homogeneity from freshly harvested spinach (*Spinacia oleracea* L.) leaves and stored at −100 °C as pellets formed from a precipitate with 50% saturated ammonium sulfate (McCurry et al., 1982). Prior to use, the precipitated enzyme was resuspended in 50 mM Bicine–NaOH, pH 8.0, containing 1 mmol of DTT. Dialysis for 12–16 h (overnight) against 1 L of 50 mM Bicine–NaOH and 0.2 mM EDTA, pH 8.0, at room temperature, followed by a brief centrifugation for clarification, yielded solutions with protein concentrations of 40–80 mg/mL. Activation of RuBP carboxylase (2 mg/mL) was allowed to proceed at room temperature for at least 30 min with 10 mM $NaHCO_3$ and 10 mM $MgCl_2$ in the assay buffer placed in a "Reacti-vial" equipped with a "Mininert-valve" (Pierce Chemical Co.). The specific activity [based on protein determined by the method of Gornall et al. (1948)] was 1.6–2.0 μmol of CO_2 fixed·min^{-1}·(mg of protein)$^{-1}$ in an assay buffer consisting of 10 mM $NaHCO_3$, 0.5 mM RuBP, 10 mM $MgCl_2$, 0.2 mM EDTA, 1 mM DTT, and 0.1 M Bicine–NaOH, pH 8.3 at 30 °C. RuBP was synthesized enzymically from ribose 5-phosphate (Horecker et al., 1958). $NaH^{14}CO_3$ was purchased from ICN Chemical and Radioisotope Division and was adjusted to 0.16 Ci/mol for standard assays or to 1.9 Ci/mol for assays in the

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[1] Abbreviations: Bicine, *N*,*N*-bis(2-hydroxyethyl)glycine; DEPC, diethyl pyrocarbonate; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid disodium salt; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; Mes, 2-(*N*-morpholino)ethanesulfonic acid; RuBP, D-ribulose 1,5-bisphosphate; RuBP carboxylase, D-ribulose-1,5-bisphosphate carboxylase/oxygenase (EC 4.1.1.39); Da, dalton(s).

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course of K_m(RuBP) and $K_m(HCO_3^-)$ determinations. Ultrapure ammonium sulfate was purchased from Schwarz/Mann. Reagent-grade Triton X-100 from Research Products International, Mt. Prospect, IL, and reagent-grade toluene from J. T. Baker were found consistently adequate for preparing scintillation cocktails. All other chemicals were obtained from Sigma Chemical Co.

Methods

Modification of RuBP carboxylase by DEPC was performed at 15 °C. RuBP carboxylase (1.6 mg/mL) was incubated with 0.2–0.8 mM DEPC applied as a freshly prepared solution in absolute ethanol. The final concentration of ethanol was 2% (v/v). DEPC concentrations were calibrated with imidazole (Miles, 1977). Mes, Hepes, and Bicine were used as buffers at 0.1 M concentrations. The ionic strength was adjusted to 0.1 with KCl. At indicated times, aliquots of 100 μL were withdrawn and diluted into 100 μL of activation buffer (20 mM $NaHCO_3$, 20 mM $MgCl_2$, 0.2 mM EDTA, 1 mM DTT, 10 mM imidazole, and 0.2 M Bicine–NaOH, pH 8.3 at 30 °C) in a 0.5-mL snap-cap container. After a 45-min incubation period at 30 °C, residual enzyme activity was determined and compared to that of a control sample.

Titration of Sulfhydryl Groups. At the end of an inactivation experiment, a 0.6-mL aliquot of the enzyme was passed through a Sephadex G-50 column (3-mL bed volume) by centrifugation (Penefsky, 1977) to remove DTT. A volume of 0.3 mL of the eluate was mixed in a cuvette with 0.6 mL of saturated urea solution, containing 1.6% (w/v) sodium dodecyl sulfate and 2 mM 5,5′-dithiobis(2-nitrobenzoic acid). The absorbance at 412 nm was monitored with a Beckman 35 spectrophotometer in the dual-beam mode with a reference cuvette containing everything except RuBP carboxylase.

Reactivation of DEPC-modified RuBP carboxylase was attempted by incubation with 0.4 M hydroxylamine at pH 7.0, at 4 °C, for 24 h. Protein samples were freed from NH_2OH by centrifugation gel filtration (Penefsky, 1977).

Determination of ε-Amino Groups. Another aliquot of RuBP carboxylase, modified by DEPC, was incubated with 1 mM pyridoxal 5′-phosphate, which was added in 0.1 M Bicine–NaOH, pH 8.0. After 10 min in the dark, $NaBH_4$ was added, and phosphopyridoxyl residues were determined as described earlier (Paech & Tolbert, 1978).

RuBP Carboxylase Assay at 30 °C. In timed intervals, typically 15 s, 20-μL aliquots of 0.25 M $NaH^{14}CO_3$ (0.16 Ci/mol) followed by 20-μL aliquots of activated enzyme (0.8–1.6 mg/mL) were added to 460 μL of the assay buffer which included RuBP and was placed in a scintillation vial. Final concentrations were 10.4 mM $NaH^{14}CO_3$, 0.5 mM RuBP, 10 mM $MgCl_2$, 0.2 mM EDTA, 1 mM DTT, and 0.1 M Bicine–NaOH, pH 8.3 at 30 °C. Carbonic anhydrase (100 units/mL) was added to all buffer solutions above pH 7.6 to ensure rapid equilibration between CO_2 and bicarbonate. Assays were terminated after 1 min by the addition of 0.5 mL of 3 N HCl. Samples were dried at 80 °C for 12–15 min, and acid-stable ^{14}C radioactivity was determined by scintillation counting using 1 mL of water and 7 mL of a toluene–Triton X-100 cocktail (Patterson & Greene, 1965). Residual ^{14}C radioactivity in assay samples without RuBP was 30 cpm above background. Enzyme activity was determined in triplicates with a standard deviation of 2%.

pH-dependent values for Michaelis constants [V_{max}, K_m(RuBP), $K_m(HCO_3^-)$] were determined by a slightly modified assay technique. The assay buffer (220 μL) and RuBP (10 μL) were placed in 2-mL polystyrene autosampler cups. The cups were covered by a friction-fit lid having a 18-gauge hole. In timed intervals, 10 μL of $NaH^{14}CO_3$ solution followed by 10 μL of activated enzyme was introduced to give a total volume of 250 μL. Immediately thereafter, a blunt 18-gauge syringe needle connected to a 1-mL syringe was inserted through the hole in the lid. The tip of the needle was allowed to rest on the conical bottom of the cup. Prior to introduction of the needle, the syringe was charged with 0.3 mL of 3 N HCl, an air bubble was drawn up, and the syringe needle was wiped clean. The air bubble served two purposes. First, it prevented premature contact of the acid with the assay solution, and second, upon discharge of the syringe, the air bubble created a turbulence which increased the effectiveness in terminating the assay. The acidified assay solutions were transferred into 7-mL scintillation vials and, after drying, subjected to scintillation counting with 0.5 mL of water and 3.5 mL of cocktail. With two experimentors in tandem, we achieved reproducibility of assays of less than 1% standard deviation. In these experiments, the assay solutions were buffered with 0.05 M Mes, 0.05 M Hepes, and 0.1 M diethanolamine, a three-component system with essentially constant ionic strength (Ellis & Morrison, 1982). The buffer stock solution was gassed constantly with N_2. For approximate pH adjustment, 6 N HCl or CO_2-free 10 M NaOH was used. The actual pH of an assay solution was determined in a separate mixture (total volume 4.8 mL) containing all assay components except RuBP in proportional quantities, and nonlabeled bicarbonate was substituted for $NaH^{14}CO_3$.

Protein concentrations in the assay varied from 0.002 to 0.08 mg/mL, depending on pH. Concentrations of RuBP ranged from 10 to 100 μM with $NaHCO_3$ remaining constant at 10 mM, while at constant RuBP concentration (0.5 mM), concentrations of $NaHCO_3$ were varied from 0.8 to 5 mM. Each assay was done in duplicate, and each set of data consisted of at least 10 data points. For statistical analyses of velocity response to substrate concentration, a program written in Basic according to Wilkinson (1961) was used. Thus, K_m and V_{max} values with standard deviations of >10% and >4%, respectively, were discarded except at both pH extremes (above pH 9 and below pH 6) where a standard deviation of <12% for K_m was tolerated.

Effective CO_2 concentrations in assays at defined pH values were calculated by the Henderson–Hasselbalch equation. From $pK = 6.317$ at 38 °C (Shedlovsky & MacInnes, 1935), the practical pK' for a given temperature and ionic strength was derived as outlined by Ellis & Morrison (1982).

Protein Determination. The biuret method by Gornall et al. (1948) was used with bovine serum albumin as a standard. For relative protein estimates, particularly in small volumes or in dilute solutions, the method of Bradford (1976) was used. A molecular mass of 530 000 Da for RuBP carboxylase and eight active sites per holoenzyme were used for all calculations.

RESULTS

Reaction with Diethyl Pyrocarbonate. The rate at which RuBP carboxylase (1.6 mg/mL) was inactivated by 0.5 mM DEPC in 0.1 M Hepes, pH 7.0 at 15 °C, was independent of the level of activation by CO_2 and Mg^{2+}, in agreement with earlier observations (Saluja & McFadden, 1982). Upon interaction with DEPC, the enzyme either may lose, in an all or none fashion, its catalytic competence or may change its kinetic parameters such that an overall lower enzymatic activity results. Partially inactivated RuBP carboxylase will have in the first case a reduced V_{max} but a constant K_m and in the latter case maybe a reduced V_{max} but always an altered K_m. DEPC affects only V_{max} (data not shown). The formation of a kinetically altered RuBP carboxylase by DEPC can be

discounted because the assay is sufficiently accurate (see Experimental Procedures) in detecting the presence of enzyme molecules with even a 100-fold higher K_m(RuBP). If the residual enzyme activity, assayed at 10 μM RuBP, represents the sum of unaltered enzyme remaining and that of enzyme with increased K_m, rather than unmodified enzyme plus totally inactive enzyme, the measured residual enzyme activity at 100 μM RuBP would be approximately 15% higher in the first case compared to that of the latter case.

The interaction of DEPC with RuBP carboxylase was investigated in Mes and Hepes buffer at constant ionic strength (I = 0.1) as a function of pH. Activation by CO_2 and Mg^{2+} was omitted to obtain the molecular pK_a value of the histidine group at the active site of the unperturbed enzyme.

Inactivation of RuBP carboxylase (1.6 mg/mL) with 0.2–0.8 mM DEPC at pH 6.2–7.4 (15 °C) did not result in measurable quantities of modified ϵ-amino and sulfhydryl groups. The total number of accessible ϵ-amino groups was measured by Schiff base formation (Paech & Tolbert, 1978). Sulfhydryl groups were titrated with 5,5′-dithiobis(2-nitrobenzoic acid) after denaturation of the enzyme with 6 M urea and 1% (w/v) sodium dodecyl sulfate (data not shown).

To follow rates of inactivation, we had two options. (a) Remove an aliquot of the enzyme–DEPC mixture at indicated times and dilute with a large volume of assay buffer containing all ingredients for the assay, except RuBP, and imidazole to quench DEPC. The enzyme would be activated for 30–45 min at almost final assay concentration. This protocol would allow one assay per time point. The residual enzyme activity would be a composite of chemical modification, activation by CO_2, and catalysis. Since activation and catalysis are affected by a single concentration of CO_2, control of CO_2 concentration and of isotopic dilution of $NaH^{14}CO_3$ by air is highly important. (b) Remove a large aliquot of the enzyme–DEPC mixture at indicated times and dilute only 1:1 with activation buffer containing sufficient imidazole to quench DEPC instantaneously. Activate the enzyme in a tightly sealed container for 30–45 min and, then, determine residual enzyme activity in small aliquots. The latter technique was chosen because it allows for an easier control of assay conditions and for a multiplicity of assays for each point taken during the time course of inactivation. We established also that the degree of activation after 45 min at 30 °C is essentially independent of the pH of the buffer solution between pH 7.2 and pH 9.2 (data not shown). This was an important control experiment because enzyme–DEPC solutions (with pH values ranging from 6.2 to 7.4), when mixed with activation buffer, will arrive at different final pH values (typically 7.7–8.0).

Pseudo-first-order kinetics were observed for inactivation of RuBP carboxylase (1.6 mg/mL) when incubated with 0.2–0.8 mM DEPC at pH 7.4 and at 15 °C (Figure 1). Because the rate of hydrolysis of DEPC was significant (Gomi & Fujioka, 1983), residual enzyme activity was plotted as function of $(1 - e^{-k't})/k'$,k' being the first-order rate constant for the hydrolysis of DEPC. This constant was determined in separate experiments and, at pH 6.2–7.4 (15 °C), was found to be 0.015–0.02 min^{-1} (data not shown). When this correction was used for the time axis, semilogarithmic plots of residual enzyme activity gave straight lines at all pH values. Pseudo-first-order rate constants for inactivation, k_{app}, were proportional to DEPC concentrations (Figure 2), and the Hill coefficient for DEPC was, on average, 1.02 ± 0.06 (cf. Figure 1, inset).

A replot of the observed pseudo-first-order rate constants of inactivation, k_{app}, at pH 6.2–7.4 is shown in Figure 2. The

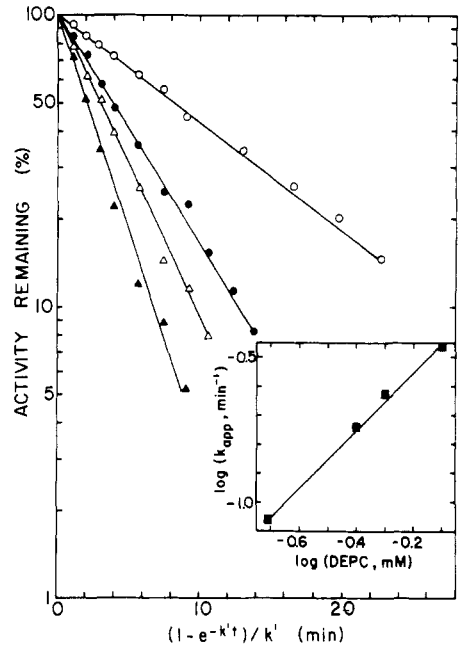

FIGURE 1: Rate of inactivation of RuBP carboxylase by DEPC. The enzyme (1.6 mg/mL) was incubated in 0.1 M Hepes at pH 7.4 (15 °C) with 0.2 (○), 0.4 (●), 0.5 (△), and 0.8 (▲) mM DEPC. Aliquots were withdrawn at timed intervals and assayed for enzyme activity. Percent activity remaining was plotted as function of $(1 - e^{-k't})/k'$ rather than actual time of sampling. This took into account, during the period of observation, the loss of DEPC due to hydroysis which proceeded with a pseudo-first-order rate constant of k' = 0.015–0.02 min^{-1}. Inset: Hill plot of observed pseudo-first-order rate constants of inactivation, k_{app}, as function of DEPC concentration. Hill coefficient n = 1.02.

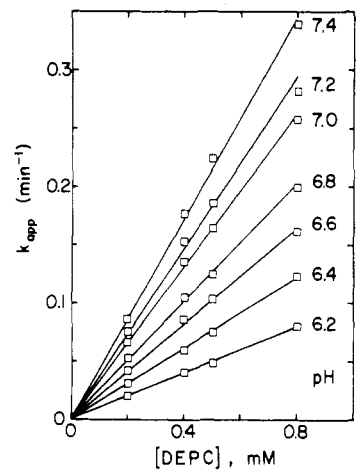

FIGURE 2: Effect of pH on observed pseudo-first-order rate constants of inactivation, k_{app}, as a function of DEPC concentration at the indicated pH (15 °C). 0.1 M Mes was used for pH 6.2–6.8 and 0.1 M Hepes for pH 7.0–7.4.

increase of inactivation rates with increasing pH is in agreement with the nonprotonated form of histidine being the reactive species in the ethoxyformylation reaction (Miles, 1977). Thus, k_{app} can be related to k_a and the proton concentration of the reaction medium as shown by Burstein et al. (1974):

$$k_{app} = k_{max}/(1 + [H^+]/K_a) \quad (1)$$

In linearized form, eq 1 becomes

$$k_{app}[H^+] = K_a k_{max} - K_a k_{app} \quad (2)$$

where k_{max} represents the pH-independent pseudo-first-order

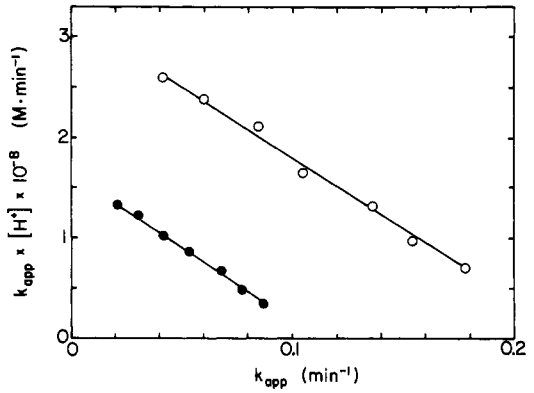

FIGURE 3: Observed pseudo-first-order rate constants, k_{app}, times proton concentration as a function of k_{app} for 0.2 (●) and 0.4 (○) mM DEPC (see Figure 2). The slopes of the lines were 1.46×10^{-7} and 1.37×10^{-7} M which gave an average pK_a = 6.85.

rate constant of inactivation. Data from Figure 2 were replotted for 0.2 and 0.4 mM DEPC and yielded pK_a = 6.85 and k_{max} values of 0.11 and 0.23 min^{-1}, respectively (Figure 3). The pH-independent second-order rate constant for inactivation was calculated to be 560 M^{-1} min^{-1}.

The experimentally obtained pK_a value was corrected for differences in temperature and ionic strength (Ellis & Morrison, 1982). d(pK_a)/dT = −0.02 for imidazole was considered a sufficiently accurate approximation. At 30 °C and ionic strength I = 0.14, the pK_a was found to be 6.54.

pH Dependence of RuBP Carboxylase Activity. A study of the pH dependence of kinetic parameters of RuBP carboxylase was conducted to see whether an ionizable group with a pK_a similar to that determined with DEPC could be discovered. One of the likely steps in which a histidine residue may play a role in the catalytic mechanism of RuBP carboxylase is the base-catalyzed proton abstraction at C-3 of RuBP, leading to the enediol/carbanion intermediate of RuBP (Calvin, 1954). One would expect, therefore, this to be expressed in pH profiles of V_{max} and V_{max}/K_m with RuBP rather than CO_2 as variable substrate.

To adjust proton concentrations from pH 5.5 to 9.3, a mixture of Mes, Hepes, and diethanolamine buffers was used. This three-component system required very little KCl to maintain constant ionic strength over the entire pH range (Ellis & Morrison, 1982). Control experiments with single-component buffer (e.g., Bicine) at identical pH and ionic strength revealed no significant differences in the kinetic parameters measured for the three-component system (data not shown).

Ideally, effective CO_2 concentrations of the assay solutions should have been constant and, preferably, saturating for RuBP carboxylase over the entire pH range under investigation since CO_2 is the substrate in the carboxylation reaction (Cooper et al., 1969). The requirement for constant ionic strength, however, did not allow an adjustment of the bicarbonate concentration with increasing pH in order to compensate for the pH-dependent decrease in CO_2 concentration. Therefore, it became necessary to establish for a constant bicarbonate concentration (10 mM) the ratio of effective CO_2 concentration and $K_m(CO_2)$ for RuBP carboxylase at any given pH. A pH dependence of $K_m(CO_2)$ for RuBP carboxylase was known (Badger, 1980) but had to be redetermined for the particular reaction conditions of the present work. Figure 4 shows log $K_m(CO_2)$ calculated from the experimentally determined $K_m(HCO_3^-)$ as a function of pH. This graph was used to normalize V_{max} values (see below).

The V_{max} and V_{max}/K_m profiles for RuBP as variable substrate as a function of pH were plotted in the usual log form

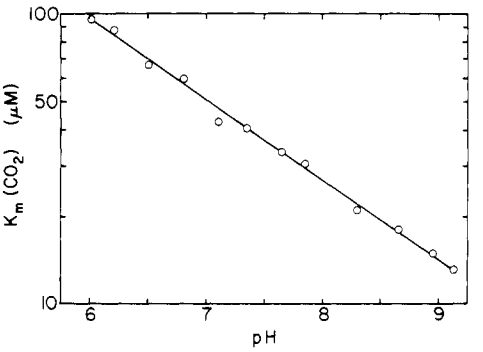

FIGURE 4: Response of $K_m(CO_2)$ to pH for RuBP carboxylase activity at 0.8 mM RuBP, 30 °C, and ionic strength I = 0.14. Details are described under Experimental Procedures. $K_m(CO_2)$ was calculated from $K_m(HCO_3^-)$ by the Henderson–Hasselbalch equation. HCO_3^- refers to the total of all species in the bicarbonate equilibrium. A thermodynamic value of pK = 6.317 at 38 °C (Shedlovsky & MacInnes, 1935) was used and corrected to 30 °C and I = 0.14 (Ellis & Morrison, 1982).

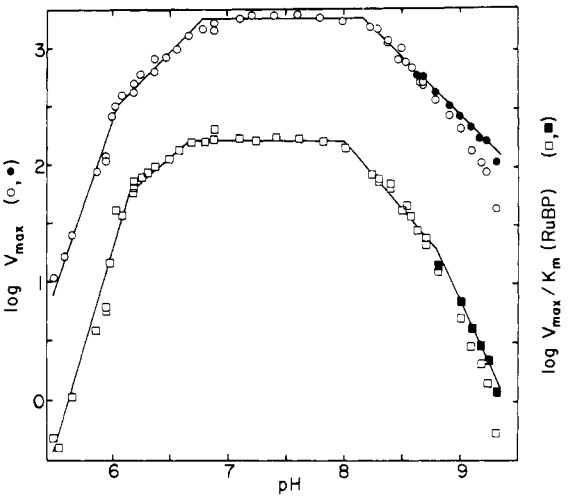

FIGURE 5: Response of V_{max}(RuBP) and of V_{max}/K_m(RuBP) to pH for RuBP carboxylase activity at 10 mM $NaHCO_3$, 30 °C, and ionic strength I = 0.14. Details of assays and data analysis are described under Experimental Procedures and under Discussion.

(Tipton & Dixon, 1979) and are shown in Figure 5. The complexity of the profiles is obvious. Their full interpretation is not possible without further experimentation (e.g., determination of pK values of RuBP). However, clearly discernible in both plots is a molecular pK of approximately 6.5–6.8. A slope of +1 on the acidic side of the break point reflects an ionizing group which participates in its unprotonated form in catalysis. A likely candidate for this function would be a histidyl residue.

On the alkaline side, the original data points (Figure 5, open symbols) did not form a straight line. In this pH range, the effect of decreasing CO_2 concentrations (at constant bicarbonate concentration) on the apparent V_{max} could not be neglected. When normalized for a constant ratio of CO_2 concentration and $K_m(CO_2)$ using the calibration curve derived from Figure 4, the corrected data produced a straight line (closed symbols, Figure 5).

Discussion

Previous reports have shown that DEPC modified one essential histidine residue per large subunit of RuBP carboxylase (Saluja & McFadden, 1980, 1982; Bhagwat & Ramakrishna, 1981). The authors have used several arguments to imply a role of this histidyl residue in the catalytic mechanism of RuBP

carboxylase. To corroborate this point, we examined whether the pH profile of kinetic parameters of RuBP carboxylase indicates a pK_a value similar to the molecular pK_a value of the DEPC-reactive histidine group.

The success in exploiting the kinetics of a chemical modification reaction for obtaining a thermodynamic parameter depends on the specificity of the reagent. DEPC is known to react with a variety of nucleophilic amino acid residues (Miles, 1977), and the relative rates will vary for different proteins. Hence, reaction conditions must be tailored such that the reactivity of histidyl residues toward DEPC is significantly higher than that of any other amino acid residue. Low temperature and acidic pH favor this requirement. However, since it is the pH dependence of ethoxyformylation which is of interest, data collection only at pH values below the expected pK'_a will give unsatisfactory results. At pH values above 7 and at elevated temperature, modification of sulfhydryl and ϵ-amino groups, to which RuBP carboxylase would be especially sensitive, is of particular concern. At pH 6.2–7.4 and 15 °C, no such side reactions were detectable by titration with 5,5′-dithiobis(2-nitrobenzoic acid) (ΔA^{1cm}_{412} = 0.026 per SH group) and pyridoxal 5′-phosphate (ΔA^{1cm}_{325} = 0.011 per ϵ-amino group). Although the absence of DEPC-modified sulfhydryl groups had been inferred from spectral studies at 230–240 nm and from comparison with the reactivity of a model compound, *N*-acetylcysteine (Saluja & McFadden, 1980), direct titration of SH groups over the entire pH range seemed appropriate.

Identical rates of inactivation in either the presence or the absence of bicarbonate/Mg^{2+} (i.e., for the activated and nonactivated enzymes, respectively) argue against a modification of the activator lysine-201 (Lorimer, 1981) by DEPC. However, failure to recover more than 80% and 95% of enzymic activity by hydroxylamine treatment after inactivation at pH 6.8 and 6.2, respectively (data not shown), remains a disturbing fact. If we assume for the sake of argument that this irreversible loss of 5–20% of enzymatic activity is due to an acylation of an ϵ-amino group which contributes to the catalytic activity, rather than a double acylation of the histidine residue, the apparent pseudo-first-order rate constants, k_{app}, will be overestimated by 4–13% at pH 6.2 and 6.8, respectively. Entered into eq 2 and Figure 3, this would result in a pK_a' of 6.72, and adjusted for ionic strength and temperature, of 6.41, which is still in favorable agreement with data obtained from the pH profile.

Hydrolysis of DEPC during the ethoxyformylation of proteins has been considered only in a few cases (Gomi & Fujioka, 1983; Hennecke & Plapp, 1983). Although, in the case of RuBP carboxylase, hydrolysis of DEPC was indicated (cf. Paech (1984)], two reports claimed noncovalent complex formation prior to covalent modification (Saluja & McFadden, 1980; Paech, 1984). When corrected for the rate of hydrolysis, k_{app} became directly proportional to the reagent concentration, and saturation kinetics could not be verified. Likewise, the Hill coefficient of 0.86 (Paech, 1984) improved to 1.02.

The breaks in the pH profile of log V_{max} indicate two ionizable groups in the enzyme–substrate complex, one with pK_a = 8.15 which must be in its protonated form during catalysis. This is qualitatively in agreement with data obtained by Christeller (1982) on the RuBP carboxylase of soybean leaves. In addition, however, Figure 5 reveals two more ionizable groups at pH 6.05 which must be in their unprotonated form for catalysis to proceed and could be attributed to substrate ionization (phosphate groups of RuBP). A slope of +3 in the pH profile of log $[V_{max}/K_m(RuBP)]$ with a break point at pH 6.05 supports this interpretation. A change of the slope from +1 to 0 (Figure 5) at pH 6.55 indicates an ionizable group of the substrate-free enzyme. This is in excellent agreement with the pK_a calculated for the histidine residue involved in DEPC inactivation (pK_a = 6.54) and strongly suggests that this group, indeed, is needed in its unprotonated form during catalysis of RuBP carboxylation. Furthermore, upon substrate binding, the pK_a value increases approximately 0.2 unit. Likewise, the pK_a = 8.05 increases upon substrate binding too.

In contrast to Christeller's data for log $[V_{max}/K_m(CO_2)]$, the pH-dependent profile of log $[V_{max}/K_m(RuBP)]$ breaks at pH 8.05 from slope 0 to −1 and, again, at pH 8.75 from slope −1 to −2. Without detailed data on the effect of other parameters, such as Mg^{2+} concentration, temperature, or organic solvents, on pH profiles of kinetic constants, however, further interpretation of the kinetic data is not possible.

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Registry No. RuBP carboxylase, 9027-23-0; DEPC, 1609-47-8; RuBP, 24218-00-6; CO_2, 124-38-9; HCO_3, 71-52-3; histidine, 71-00-1.

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Interaction of Bleomycin A_2 with Deoxyribonucleic Acid: DNA Unwinding and Inhibition of Bleomycin-Induced DNA Breakage by Cationic Thiazole Amides Related to Bleomycin A_2†

L. Mark Fisher*
Department of Biochemistry, St George's Hospital Medical School, University of London, Cranmer Terrace, London SW17 ORE, U.K.
Reiko Kuroda
CRC Biomolecular Structure Unit, Institute of Cancer Research, Sutton, Surrey SM2 5PX, U.K.
Ted T. Sakai
Comprehensive Cancer Center and Department of Biochemistry, University of Alabama in Birmingham, Birmingham, Alabama 35294
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ABSTRACT: The association of the antitumor antibiotic bleomycin A_2 with DNA has been investigated by employing several 2-substituted thiazole-4-carboxamides, structurally related to the cationic terminus of the drug. With a 5′-^{32}P-labeled DNA restriction fragment from plasmid pBR322 as substrate, these compounds have been shown to inhibit bleomycin-induced DNA breakage. Analogues possessing 2′-aromatic substituents on the bithiazole ring were more potent inhibitors than those carrying 2′-aliphatic groups, e.g., the acetyl dipeptide A_2. The degree of inhibition was similar at all scission sites on DNA, and inclusion of the analogues did not induce bleomycin cleavage at new sites. DNA binding of bithiazole derivatives has also been studied by two complementary topological methods. Two-dimensional gel electrophoresis using a population of DNA topoisomers and DNA relaxation experiments involving calf thymus DNA topoisomerase I and pBR322 DNA reveal that bleomycin bithiazole analogues unwind closed circular duplex DNA. The inhibition and unwinding studies together support recent NMR studies suggesting that both bleomycin A_2 and synthetic bithiazole derivatives bind to DNA by an intercalative mechanism. The results are discussed in relation to the DNA breakage properties of bleomycin A_2.

The bleomycins are a group of glycopeptide antitumor antibiotics that are used clinically in the treatment of certain cancers [for reviews, see Hecht (1979) and Povirk (1983)]. Their antitumor activity is thought to arise from their ability to degrade DNA. Bleomycins bind and are activated by a variety of transition metal ions including Fe(II). Studies in vitro have demonstrated that the bleomycin–Fe(II) complex forms a redox-active species with O_2 that mediates DNA degradation (Ishida & Takahashi, 1975; Horwitz et al., 1979; Burger et al., 1981).

There is considerable evidence that the bleomycin–Fe(II) complex interacts in a specific manner with double-stranded DNA causing the release of DNA bases (Haidle et al., 1972; Muller et al., 1972; Ishida & Takahashi, 1975; Povirk et al., 1978; Sausville et al., 1978) and the introduction of single- and double-stranded DNA breaks (Suzuki et al., 1968; Nagai et al., 1969; Shirakawa et al., 1971). Thus, although all four bases are released from DNA, bleomycin preferentially liberates thymine > cytosine > adenosine > guanine (Povirk et al., 1978; Sausville et al., 1978). Secondly, DNA strand scission occurs at a limited number of sites with preferential cleavage at GC and GT sequences (D'Andrea & Haseltine, 1978; Takeshita et al., 1978, 1981; Kross et al., 1982a; Mirabelli et al., 1982). The molecular basis underlying DNA specificity exhibited by bleomycins remains to be elucidated.

Several groups have shown that the bleomycin molecule is composed of two functionally distinct domains, shown in Figure 1 for bleomycin A_2, the most common congener (Chien et al., 1977; Kasai et al., 1978; Takita et al., 1978; Glickson et al.,

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