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Kinetic Properties of Cyanase†

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ABSTRACT: Cyanase is an inducible enzyme in *Escherichia coli* that catalyzes the hydrolysis of cyanate. Bicarbonate is required for activity, perhaps as a substrate, and the initial product of the reaction is carbamate, which spontaneously breaks down to ammonia and bicarbonate [Anderson, P. M. (1980) *Biochemistry 19*, 2882]. The purpose of this study was to characterize the kinetic properties of cyanase. Initial velocity studies showed that both cyanate and bicarbonate act as competitive substrate inhibitors. A number of monovalent anions act as inhibitors. Azide and acetate appear to act as competitive inhibitors with respect to cyanate and bicarbonate, respectively. Chloride, bromide, nitrate, nitrite, and formate also inhibit, apparently as the result of binding at either substrate site. Malonate and several other dicarboxylic dianions at very low concentrations display "slow-binding", reversible inhibition which can be prevented by saturating concentrations of either substrate. The results are consistent with a rapid equilibrium random mechanism in which bicarbonate acts as a substrate, bicarbonate and cyanate bind at adjacent anion-binding sites, and both substrates can bind at the other substrate anion binding site to give a dead-end complex.

Cyanase is an inducible enzyme in *Escherichia coli* that catalyzes the hydrolysis of cyanate to give ammonia and bicarbonate. The enzyme has been highly purified in our laboratory, and several unusual and interesting aspects of the structural and kinetic properties have been identified (Anderson, 1980). The presence of bicarbonate is required for catalytic activity. The initial product of the reaction is carbamate, which spontaneously breaks down to bicarbonate and ammonia. The enzyme has a molecular weight of $\simeq$150000 and is composed of 8–10 identical subunits. Amino acid analysis and sequence studies have shown that the cyanase subunit (M_r 16750) is composed of 156 amino acid residues (one histidine, one cysteine, and no tryptophan residues) with no evidence for any of the common secondary structural features (Chin et al., 1983).

We have initiated studies aimed at elucidating the nature of the catalytic mechanism, the role of bicarbonate, and the biological role of this unusual enzyme. The purpose of this study was to characterize the kinetic properties of cyanase. A preliminary report has appeared (Anderson & Little, 1985).

Materials and Methods

Cyanase was isolated as previously described (Anderson, 1980). Cyanate was recrystallized from a water–ethanol mixture before use. Other reagents were obtained from Sigma Chemical Co.

Assay. Kinetic studies with this enzyme are complicated by several features of the reaction system. It is very difficult and impractical to prepare buffers totally free of low concentrations of bicarbonate. Cyanate is labile in aqueous solutions at pH 6–7 and lower due to hydrolysis of the protonated form of cyanate (Labbe, 1973), and highly purified cyanate

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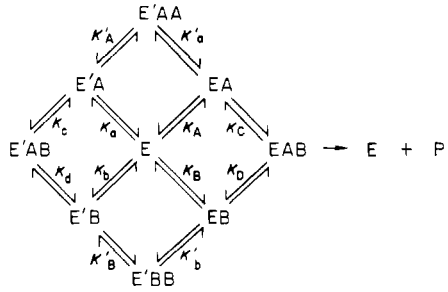
Scheme I



(recrystallized) contains some bicarbonate and ammonia (≃0.03% in our best preparations). To minimize these difficulties and sources of errors, assays were carried out in 0.05 M potassium phosphate buffer, pH 6.8, equilibrated with atmospheric CO_2. A pH of 6.8 is low enough so that endogenous bicarbonate is minimal, is high enough for satisfactory cyanate stability, and is near the optimal pH of 7.2 for catalytic activity. Endogenous bicarbonate was estimated to be 0.07 mM on the basis of calculation and by adjustment of the values of the added variable bicarbonate concentrations until linear double-reciprocal plots of rate vs. bicarbonate concentration were obtained under conditions where linear plots were expected (McClure et al., 1971). Assay mixtures were prepared just prior to use.

Rates (μmol of ammonia/mL formed per 11 min) were determined by measuring the ammonia concentration after 11 min at 26 °C. To assure that the rates obtained by measuring ammonia at 11 min reflected initial velocity rates, conditions were chosen so that the concentration of ammonia was not greater than about 5–8% of the initial substrate concentration. Ammonia was determined by a modification of the colorimetric procedure described by Koops et al. (1975) utilizing dichloroisocyanurate and salicylate. The reaction was stopped by adding 0.2 mL of a solution containing 1.44 M sodium salicylate, 5.5 mM sodium nitroprusside, and 3 M NaCl (cyanase is competitively inhibited by chloride). An equal volume (0.2 mL) of a solution containing 0.031 M sodium dichloroisocyanurate and 2.7 M KOH was then added with immediate mixing. After 30 min at 26 °C (or 15 min at 40 °C) the A_{660} was measured with a Beckman DU-8 spectrophotometer equipped with a sipper-cell system. The modified procedure was sensitive to concentrations of ammonia as low as 1 μM.

Analysis of Kinetic Data. Data from initial velocity studies were first analyzed graphically by double-reciprocal plots of initial velocities vs. concentration of cyanate or bicarbonate. Linear plots were fitted to eq 1, while plots displaying substrate

$$v = \frac{VA}{K + A} \tag{1}$$

$$v = \frac{VA}{K + A + A^2/K_i} \tag{2}$$

inhibition were fitted to eq 2 using the Fortran programs of Cleland (1979). Changes in the slopes and intercepts of the double-reciprocal plots as a function of the concentration of the nonvarying substrate, and other data, were further interpreted on the basis of the following rapid equilibrium random mechanism (Scheme I) in which A or B (bicarbonate and cyanate) can each act as dead-end inhibitors by binding at the B or A binding sites, respectively (i.e., substrate inhibition); E′ represents an inactive enzyme form in which A, B, or both are bound at the wrong site. The rate equations describing this mechanism, arranged in double-reciprocal form for $1/v$ vs. $1/A$ (eq 3) or $1/B$ (eq 4), are

$$\frac{V}{v} = \frac{1}{A}\left(K_D + \frac{K_BK_D}{K_b} + \frac{K_AK_C}{B} + \frac{BK_D}{K'_b}\right) + \left[1 + \frac{K_AK_C}{K_aK_c} + \frac{1}{B}\left(K_C + \frac{K_AK_C}{K_a}\right) + \frac{AK_C}{BK'_a}\right] \tag{3}$$

$$\frac{V}{v} = \frac{1}{B}\left(K_C + \frac{K_AK_C}{K_a} + \frac{K_AK_C}{A} + \frac{AK_C}{K'_a}\right) + \left[1 + \frac{K_AK_C}{K_aK_c} + \frac{1}{A}\left(K_D + \frac{K_BK_D}{K_b}\right) + \frac{BK_D}{AK'_b}\right] \tag{4}$$

In each case, the nonvarying substrate (at *higher concentrations*) gives rise to competitive substrate inhibition. The slope of double-reciprocal plots would increase linearly with increasing concentration of the nonvarying substrate. Under these conditions, therefore, secondary plots of slopes vs. nonvarying substrate concentration give values for the following kinetic constants (groups of equilibrium constants):

$$K_D + \frac{K_BK_D}{K_b} \qquad \frac{K_D}{K'_b} \qquad K_C + \frac{K_AK_C}{K_a} \qquad \frac{K_C}{K'_a}$$

At *lower concentrations* of nonvarying substrate, however, the double-reciprocal plots would be nonlinear due to substrate inhibition by the varying substrate. Under these conditions the data can be fitted to eq 2 by using the computer program described above, which gives the slope and intercept of the asymptote to the curve at very low concentrations of varying substrate (i.e., the slope and intercept which would be obtained if substrate inhibition was not occurring, eliminating the last term in eq 3 and 4). Under these conditions the slope would begin to increase with decreasing concentration of nonvarying substrate. An estimate of K_AK_C ($=K_BK_D$) can be made from the values of the concentrations of the nonvarying substrate at which the slope reaches a minimum. The intercept (also determined by using eq 2 as described) at the lower concentrations of nonvarying substrate would be linearly related to the reciprocal of the concentration of the nonvarying substrate. Thus, under these conditions, plots of intercepts vs. reciprocal of the nonvarying substrate concentration give values for the following kinetic constants (groups of equilibrium constants):

$$1 + \frac{K_AK_C}{K_aK_c} \qquad K_D + \frac{K_BK_D}{K_b} \qquad K_C + \frac{K_AK_C}{K_a}$$

The progress curve for the time-dependent inhibition by malonate was fitted to eq 5 as described by Morrison (1982) for slow-binding inhibitors. The symbols v_0, v_s, t, k, and p, respectively, represent initial velocity, steady-state velocity, time, an apparent first-order rate constant, and concentration of product.

$$P = v_s t + (v_0 - v_s)(1 - e^{-kt})k \tag{5}$$

Results

Initial Velocity Kinetics. Double-reciprocal plots of initial velocity vs. bicarbonate concentration are shown in Figures 1 and 2 at different lower and higher concentrations of the nonvarying substrate cyanate, respectively. The data in Figure 1 is fitted to eq 2, indicating substrate inhibition by bicarbonate. The results in Figure 2 indicate that cyanate can also give rise to substrate inhibition, acting in this case (nonvarying substrate) as a competitive substrate inhibitor. Secondary plots of intercept vs. the reciprocal of cyanate

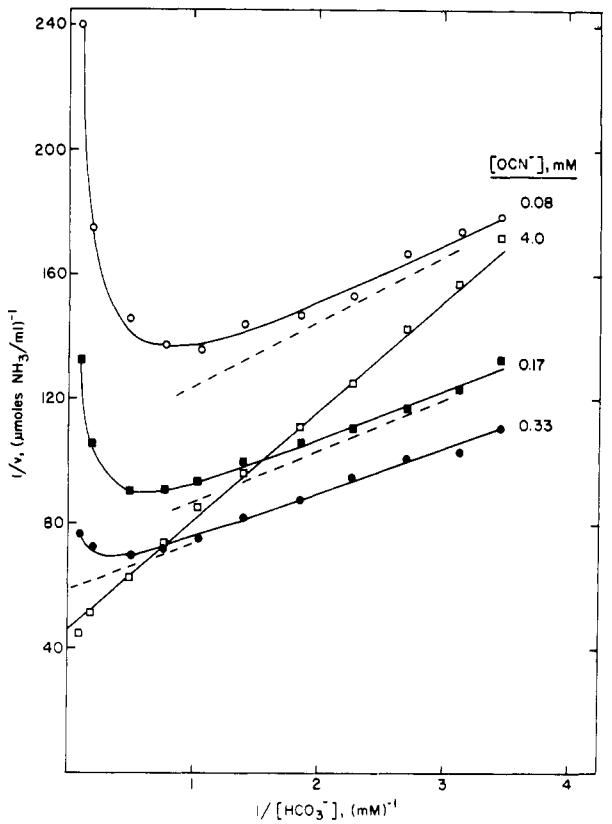


FIGURE 1: Double-reciprocal plots showing substrate inhibition by bicarbonate at different lower concentrations of the nonvarying substrate cyanate. The dashed lines are the asymptotes determined from eq 2.

Table I: Values of Kinetic Constants Calculated from Secondary Plots

kinetic constant	value (mM)	experimental parameters[a] varying substrate	secondary plot
$K_D + K_BK_D/K_b$	0.23 ± 0.03	bicarbonate	slopes
$K_D + K_BK_D/K_b$	0.37 ± 0.13	cyanate	intercepts
$K_C + K_AK_C/K_a$	0.14 ± 0.02	cyanate	slopes
$K_C + K_AK_C/K_a$	0.12 ± 0.01	bicarbonate	intercepts
$1 + K_AK_C/(K_aK_c)$	1.01 ± 0.03	bicarbonate	intercepts
$1 + K_AK_C/(K_aK_c)$	0.98 ± 0.05	cyanate	intercepts
K_D/K'_b	0.20 ± 0.04	bicarbonate	slopes
K_C/K'_a	0.019 ± 0.003	cyanate	slopes

[a] The two columns indicate whether the values for the kinetic constants were determined from secondary plots of slopes or intercepts and whether the secondary plots were from double-reciprocal plots with bicarbonate or cyanate as the varying substrate.

concentration from Figure 1 and of slope vs. cyanate concentration from Figure 2 are shown in Figure 3. The secondary plot of slope vs. cyanate concentration shows that the slope decreases linearly with decreasing cyanate concentration but begins to increase at very low concentrations of cyanate, as would be expected according to eq 3 and 4. Analogous results are obtained when cyanate is the variable substrate and bicarbonate is the nonvarying substrate; substrate inhibition by cyanate occurs at lower concentrations of bicarbonate, bicarbonate acts as a competitive substrate inhibitor at higher concentrations of bicarbonate, and the corresponding secondary plots are linear (plot of slope vs. cyanate concentration becomes nonlinear at very low concentrations of cyanate, as expected).

The values for the different kinetic constants (groups of equilibrium constants) associated with eq 3 and 4 based upon Scheme I and determined from the secondary plots as described under Materials and Methods are given in Table I.

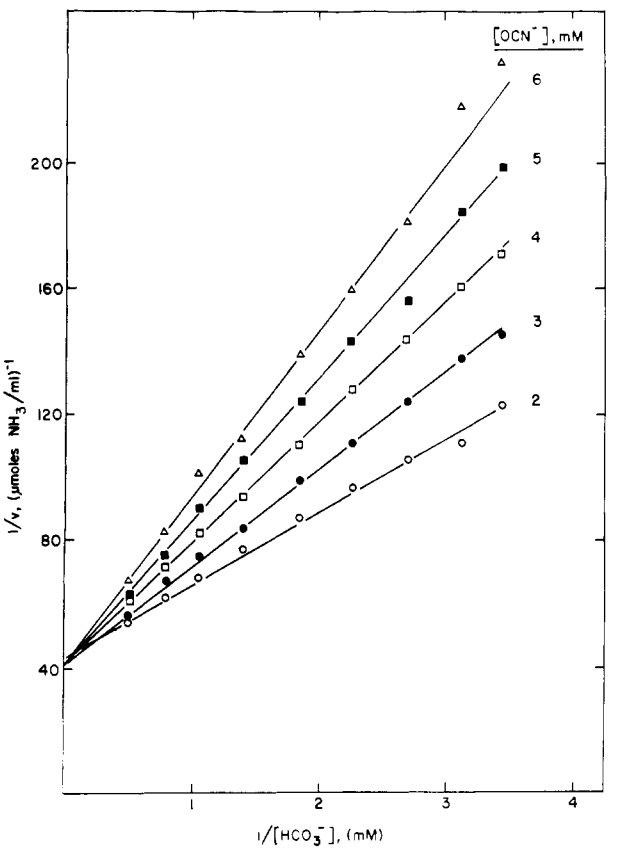


FIGURE 2: Double-reciprocal plots showing competitive substrate inhibition by different higher concentrations of the nonvarying substrate cyanate.

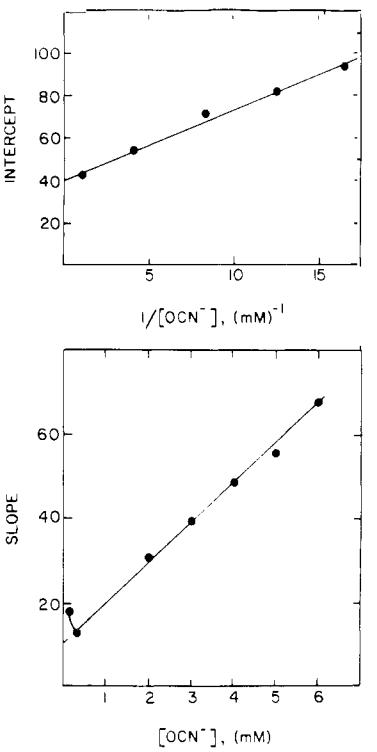


FIGURE 3: Secondary plots of intercepts and slopes from Figures 1 and 2, respectively.

As noted, the sets of two values for $K_D + K_BK_D/K_b$, $1 + K_AK_C/(K_aK_c)$, and $K_C + K_AK_C/K_a$ obtained from the two different double-reciprocal and secondary plots are in reasonably close agreement. Also, the finding that the value of $1 + K_AK_C/(K_aK_c)$ [$=1 + K_BK_D/(K_bK_d)$] is essentially 1 in-

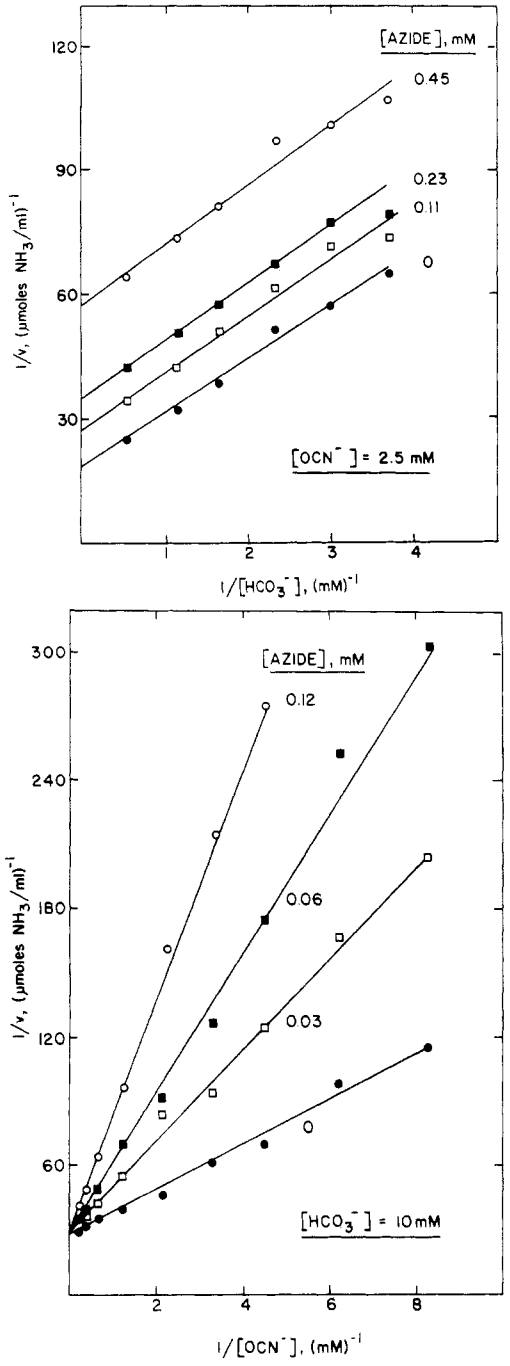


FIGURE 4: Double-reciprocal plots showing inhibition of cyanase by azide. The concentrations of azide and the nonvarying substrate are indicated.

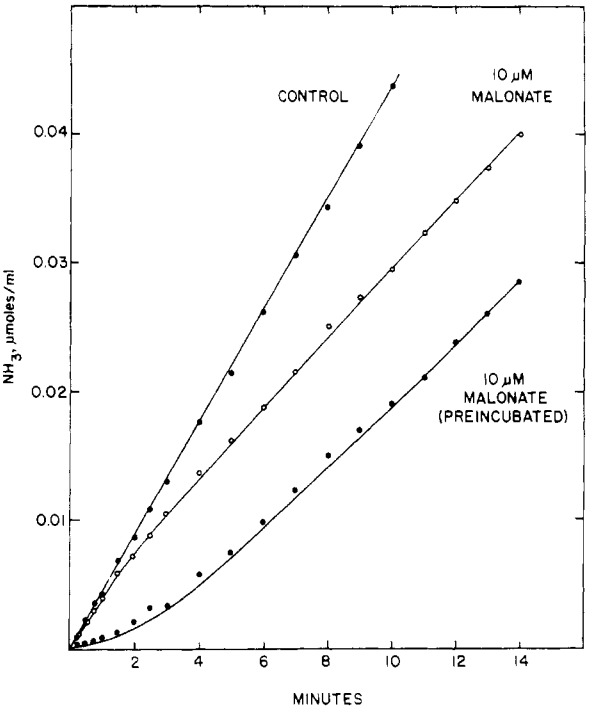


FIGURE 5: Effect of malonate on the time course of cyanase activity. The reaction mixture contained bicarbonate (0.3 mM), cyanate (0.3 mM), and, where indicated, malonate (0.01 mM) either preincubated with the enzyme for 60 min at 26 °C before adding substrates or present in the assay mixture when enzyme was added to initiate the reaction, as indicated.

dicates that the values of the equilibrium constants for the dead-end binding of substrates are significantly higher than the values of the equilibrium constants for binding at the correct substrate site, as would be expected. Consequently, the values obtained for the first two groups of equilibrium constants in Table I probably represent a reasonable approximation of the values for K_D and K_C, respectively.

Due to the limitations of kinetic assays imposed by the inherent nature of the reaction catalyzed as discussed under Materials and Methods as well as the high degree of substrate inhibition observed with relatively low concentrations of either substrate, and the relatively low apparent binding constants for the substrates, difficulties were encountered in attempts to measure initial rates under conditions where substrate inhibition by either the varying substrate or the nonvarying substrate was minimal, i.e., at low concentrations of each substrate. This was particularly manifested in efforts to obtain accurate estimates for K_AK_C ($=K_BK_D$). As described under Materials and Methods, the point at which the slope reaches a minimum in secondary plots of slope vs. nonvarying substrate concentration can theoretically be used to determine K_AK_C ($=K_BK_D$). As shown in Figure 3 and as described above, a reversal in the linear decrease in slope with decreasing nonvarying substrate concentration is observed when the latter reaches a very low value, as expected, but accurate estimates of K_AK_C ($=K_BK_D$) could not be obtained.

Inhibition by Mono- and Dianions. A number of monoanions inhibited cyanase activity, presumably by binding reversibly at the bicarbonate and/or cyanate binding sites. The exact nature of the mode of inhibition is difficult to determine due to substrate inhibition by both substrates and the consequent likelihood of binding of the monoanion inhibitors at either site. Azide appears to have relatively high specificity for the cyanate site, acting as a competitive inhibitor with respect to cyanate and as a noncompetitive inhibitor with respect to bicarbonate (Figure 4). Inhibition by azide appears to become uncompetitive with respect to bicarbonate when the concentration of cyanate is 0.2 mM or less, however. Acetate at fairly high concentrations (20–200 mM) acts as a competitive inhibitor with respect to bicarbonate and as a noncompetitive inhibitor with respect to cyanate, indicating specificity for the bicarbonate binding site. Chloride, bromide, formate, and nitrate were found to be competitive inhibitors with either cyanate or bicarbonate when the concentration of the nonvarying substrate is very high but noncompetitive with either substrate when the concentration of the nonvarying substrate is not high, suggesting that these anions probably bind to either substrate site.

Low molecular weight dicarboxylic acids are particularly effective inhibitors. As shown in Figure 5, inhibition by

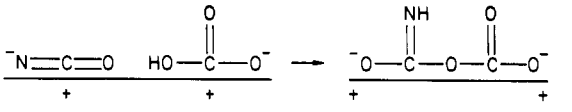
malonate is time dependent, but reversible, reaching a steady-state level of reduced rate. This reduced steady-state rate is dependent upon substrate and inhibitor concentration. When the enzyme is preincubated with malonate before addition of substrates, the initial rate reflects substantially more inhibition, but the rate increases with time to give the same approximate final steady-state level of reduced activity. The inhibition by malonate is characteristic of the class of inhibitors called slow-binding inhibitors (Morrison, 1982), and the progress curves can be fitted to eq 5 (Figure 5). The presence of either bicarbonate or cyanate at higher, saturating concentrations (3 mM) prevents significant inhibition. Oxalate and oxalacetate are more inhibitory than malonate at equal concentrations, but a much longer period of time (>1 h) is required to attain a final steady-state level of reduced rate. Preincubation of cyanase with oxalate in the presence of substrates results in a time-dependent decline in enzyme activity (measured by assaying appropriate aliquots under standard assay conditions, except that the assay time was 2 rather than 11 min; the inhibition by oxalate is reversible, but at a rate which is much slower than that observed for malonate, so that little increase in activity is observed during the 2-min assay). The rate of this decline in activity is significantly reduced by the additional presence of either azide or bicarbonate in the preincubation solution; the rate is reduced by 50% when the concentrations of azide or bicarbonate are 0.01 and 0.4 mM, respectively, and the concentration of oxalate is 0.04 mM (at pH 6.8, 26 °C). Significantly higher concentrations of maleate, malate, methylmalonate, and hydroxymalonate are required to achieve the same degree of inhibition as that observed by malonate, and phthalate, fumarate, succinate, and α-ketoglutarate are not effective inhibitors.

Discussion

The requirement for bicarbonate is an unusual feature of the catalytic properties of cyanase. Bicarbonate has been shown to be a required activator for several enzyme activities, including ribulosebisphosphate carboxylase, in which CO_2 reacts with a specific amino group to form a carbamate resulting in a change in charge which facilitates binding of a divalent cation (Lorimer & Miziorko, 1980), phosphoenolpyruvate carboxykinase ferroactivator (Merryfield, 1979), and an ATPase (Rothlein & Parsons, 1982), and specific carbamate formation with hemoglobin has physiological significance (Kilmartin & Rossi-Bernardi, 1973). It has been suggested that carbamate formation as a form of metabolic regulation might be widespread (Lorimer, 1983).

The role of bicarbonate in the catalytic mechanism of cyanase could also be that of an activator (e.g., via carbamate formation), or bicarbonate could function as a recycling substrate in which bicarbonate reacts with cyanate to give an intermediate that breaks down to carbamate and CO_2 (Anderson, 1980). The initial velocity kinetic studies described above are consistent with Scheme I, a rapid equilibrium random mechanism in which bicarbonate acts as a substrate and both substrates (bicarbonate and cyanate) display substrate inhibition by binding at the other substrate binding site to give a dead-end complex (formation of E′AA and E′BB from EA and EB, respectively). A ping-pong mechanism with substrate inhibition and a rapid equilibrium ordered mechanism are not consistent with the results. A steady-state ordered mechanism in which, for example, A binds first to give EA, A can bind to EA to give E′AA, and B can bind to E to give E′B would also show competitive substrate inhibition by both substrates, and the rate equation would have the same general form as

Scheme II



eq 3 or 4 so that the mechanisms are indistinguishable by initial velocity studies. The following additional observations provide evidence favoring the random mechanism. The observation that both azide and acetate give noncompetitive inhibition with respect to bicarbonate and cyanate, respectively, would be consistent with the random mechanism but not with the ordered mechanism if inhibition is due to fairly specific binding at the cyanate and bicarbonate sites, respectively. The observation that inhibition by azide with respect to bicarbonate at very low cyanate concentration appears to be uncompetitive, if correct, would be consistent with an ordered mechanism with bicarbonate adding first; however, the random mechanism would give the same result if azide had a greater affinity for the EA (A = bicarbonate) complex than for E under these conditions. The observation that relatively low concentrations of either azide (as a cyanate analogue) or bicarbonate significantly reduce the rate of time-dependent inhibition by oxalate is consistent with a random mechanism, although an ordered mechanism (with bicarbonate binding first) cannot be excluded on this basis without a detailed understanding of the pathway of oxalate binding. Preliminary studies have shown that cyanate is irreversibly inhibited by reaction with tetranitromethane or iodine and that inhibition by either reagent can be prevented by the presence of azide, but not bicarbonate (X. Xiong and P. M. Anderson, unpublished experiments), indicating that azide (presumably as an analogue of cyanate) can bind in the absence of bicarbonate, which would be expected for the random mechanism.

Although these studies do not rule out the possibility that bicarbonate may act as an activator without participating directly as a substrate, a mechanism in which the role of bicarbonate is that of a compulsory order activator which must bind first but does not participate directly in the catalytic mechanism, as well as a mechanism involving an activator site as well as a substrate site for bicarbonate, would not be consistent with the data.

The kinetic mechanism in Scheme I in which bicarbonate acts as a substrate would suggest two anion-binding sites, one for bicarbonate and one for cyanate. The finding that both substrates exhibit competitive substrate inhibition, that monoanions act as competitive inhibitors with varying specificity for each site depending upon the degree of structural similarity to each substrate, and that small dicarboxylic acids (dianions) are unusually effective inhibitors has provided additional support for the view that the active site is comprised of two adjacent anion substrate binding sites which accommodate a dianion intermediate formed by the reaction of bicarbonate with cyanate, as previously suggested and as illustrated in Scheme II (Anderson, 1980).

The kinetics of inhibition by malonate and related dianions, characterized by slow binding, suggest that inhibition is preceded by an initial interaction, perhaps at the bicarbonate binding site, which leads, in a slow process, to "tight" binding at the active site (Morrison, 1982). The slow process might be related to conformational changes in the monomer to accommodate dianion binding at the two adjacent anion binding sites with consequential effects on the quaternary structure of cyanase. Binding and kinetic studies with the dianion inhibitors currently in progress are expected to provide further insights into the nature of the active site, the catalytic

mechanism and role of bicarbonate, and, perhaps, the significance of the unusual quaternary structure of cyanase.

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Registry No. OCN^-, 661-20-1; HCO_3^-, 71-52-3; N_3^-, 14343-69-2; HOAc, 64-19-7; Cl^-, 16887-00-6; Br^-, 24959-67-9; HCO_2H, 64-18-6; NO_3^-, 14797-55-8; NO_2^-, 14797-65-0; $CH_2(CO_2H)_2$, 141-82-2; $(CO_2H)_2$, 144-62-7; $HO_2CCOCH_2CO_2H$, 328-42-7; cyanase, 37289-24-0.

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Spectral Characterization of the Oxidized States of Lignin Peroxidase, an Extracellular Heme Enzyme from the White Rot Basidiomycete *Phanerochaete chrysosporium*†

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ABSTRACT: Lignin peroxidase (diarylpropane oxygenase, ligninase, LiP), an H_2O_2-dependent lignin degrading heme enzyme from the basidiomycetous fungus *Phanerochaete chrysosporium*, catalyzes the oxidation of a variety of lignin model compounds. In this paper, we characterize the H_2O_2-oxidized forms of LiP compounds I, II, and III by electronic absorption spectroscopy. The native enzyme contains high-spin ferric iron and has its Soret maximum at 407.6 nm and α and β bands at 496 and 630 nm. Addition of 1 molar equiv of H_2O_2 to the native enzyme in 20 mM sodium phosphate, pH 6.0, yields compound I, characterized by a Soret maximum at 408 nm with reduced intensity and by additional maxima at 550, 608, and 650 nm. Addition of 2 molar equiv of H_2O_2 to the native enzyme yields compound II, identified by absorption maxima at 420, 525, and 556 nm. Addition of a molar excess of H_2O_2 to the native enzyme yields compound III, which is characterized by absorption maxima at 419, 540, and 578 nm. These spectral characteristics are very similar to those of horseradish peroxidases (HRPs) I, II, and III. In 20 mM sodium phosphate, pH 6.0, LiP-I spontaneously converts to LiP-II with a $t_{1/2}$ of $\sim$1.0 min, and this conversion is characterized by a single isosbestic point in the visible absorption spectrum at 565 nm. The organic peroxides *m*-(chloroperoxy)benzoic acid and *p*-(nitroperoxy)benzoic acid oxidize LiP to compounds I and II, respectively. Addition of 0.5 molar equiv of veratryl alcohol, a two-electron substrate, to LiP-I rapidly reduces it to LiP-II; further addition of 0.5 molar equiv of veratryl alcohol to LiP-II reduces it to the native ferric enzyme. This indicates that like HRP-I and HRP-II compounds I and II of lignin peroxidase contain 2 and 1 oxidizing equiv over the ferric resting state of the enzyme. It also suggests that the dehydrogenation of veratryl alcohol by this enzyme proceeds via two single-electron oxidation steps rather than through a single two-electron oxidation mechanism.

Lignin is a complex, optically inactive, heterogeneous, and random biopolymer (Sarkanen, 1971) that comprises 20–30% of woody plants. Since the biodegradation of cellulose is also retarded by the presence of lignin (Crawford, 1981), the catabolism and utilization of this intractable phenylpropanoid polymer are of enormous importance and interest. In nature, white not basidiomycetous fungi are primarily responsible for the decomposition of lignin (Crawford, 1981). Recent studies have shown that when cultured under aerobic conditions, the white rot basidiomycete *Phanerochaete chrysosporium* produces two extracellular H_2O_2-dependent enzymes (Kuwahara et al., 1984; Glenn & Gold, 1985), lignin peroxidase and an Mn(II) peroxidase.

Lignin peroxidase (ligninase, diarylpropane oxygenase, LiP)[1] has been purified to homogeneity from the extracellular me-

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[1] Abbreviations: EPR, electron paramagnetic resonance; LiP, lignin peroxidase; HRP, horseradish peroxidase; KTBA, 2-keto-4-(methylthio)butyric acid; CPO, chloroperoxidase; CAT, catalase; mCPBA, *m*-(chloroperoxy)benzoic acid; pNPBA, *p*-(nitroperoxy)benzoic acid.

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