Interaction of Mono- and Dianions with Cyanase: Evidence for Apparent Half-Site Binding†

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ABSTRACT: Cyanase is an inducible enzyme in *Escherichia coli* that catalyzes bicarbonate-dependent hydrolysis of cyanate. The dianions oxalate, oxalacetate, and malonate are slow-binding inhibitors of cyanase, and some monoanions such as azide and chloride also inhibit cyanase activity [Anderson, P. M., & Little, R. M. (1986) *Biochemistry 25*, 1621–1626]. The purpose of this study was to investigate the interaction of selected dianions and monoanions by kinetic and equilibrium dialysis binding studies in an effort to obtain information about the active site and catalytic mechanism. Measurement of the effectiveness of 30 different dianions as inhibitors of cyanase showed a significant degree of structural and/or isomeric specificity and considerable variation with respect to the slow-binding nature of the inhibition. Oxalate and oxalacetate both show extreme slow-binding inhibition at very low concentrations. Kinetic studies of the rate of inhibition of cyanase by oxalate showed that the reaction is pseudo first order with respect to oxalate concentration and the results are consistent with a pathway in which oxalate forms a complex with the enzyme in a rapid initial reversible step followed by a slow isomerization step leading to a complex with a very low dissociation constant. The rate of inhibition is significantly reduced by the presence of relatively low concentrations of either azide (analogue of cyanate) or bicarbonate. Equilibrium dialysis binding studies showed that the stoichiometry of binding at saturation for oxalate, malonate, chloride, and bicarbonate is about 0.5 mol of ligand bound/mol of subunit for each compound. Oxalate binding is greatly reduced by the presence of either azide or bicarbonate, and by other related dianions. Chloride and bicarbonate binding are both inhibited by the presence of dianions such as oxalate. Chloride binding is significantly inhibited by the presence of azide, but not by bicarbonate, and bicarbonate binding is not significantly affected by the presence of either azide or chloride, indicating that chloride (like azide) binds primarily as an analogue of cyanate and that chloride and bicarbonate can both bind to cyanase at near saturation at the same time. The results of these studies are consistent with a mechanism of inhibition by dianions that involves specific interaction with adjacent cyanate and bicarbonate substrate binding sites and support previous initial velocity kinetic studies that provided evidence for a rapid equilibrium random mechanism in which bicarbonate acts as a substrate and cyanate and bicarbonate bind at adjacent anion binding sites.

Cyanase is an inducible enzyme in *Escherichia coli* that catalyzes the hydrolysis of cyanate. The enzyme has a molecular weight of ≈150 000 and is composed of 8–10 identical subunits (M_r 17 008) (Anderson, 1980; Chin et al., 1983). Bicarbonate is required for catalytic activity, and the initial product of the reaction is carbamate, which spontaneously breaks down to bicarbonate and ammonia (Anderson, 1980). The results of kinetic studies are consistent with a 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 resulting in substrate inhibition (Anderson & Little, 1986). These studies showed that certain monoanions and dianions are very effective inhibitors of cyanase activity. Azide is a competitive inhibitor with respect to cyanate. Oxalate, oxalacetate, and, to a lesser extent, malonate at low concentrations display slow-binding, reversible inhibition that can be prevented by saturating concentrations of either cyanate or bicarbonate, suggesting that these dianions interact with cyanase by binding at both substrate anion binding sites.

A study of the properties of the interaction of dianions, the substrate bicarbonate, and several monoanions with cyanase using equilibrium dialysis and kinetic methods is reported here. The results of this investigation have shown that the stoichiometry of binding of the anions investigated is one per two subunits and suggests that the slow-binding inhibition by oxalate involves initial interaction at the active site followed by slow isomerization of the enzyme leading to a complex that has a very low dissociation constant and involves both substrate sites. The results also show that bicarbonate and analogues of cyanate can bind to the enzyme independently and also at the same time, which supports the results of kinetic studies that provided evidence favoring a rapid equilibrium random mechanism (Anderson & Little, 1986).

Materials and Methods

Cyanase was isolated essentially as previously described (Anderson, 1980), except the enzyme was induced in a strain of *E. coli* in which the gene for cyanase had been cloned resulting in a 1000-fold increase in enzyme production.[1] Procedures for cloning cyanase will be reported elsewhere. Cyanate was recrystallized from a water–ethanol mixture before use.

Cyanase activity was assayed as previously described (Anderson & Little, 1986). The standard assay mixture contained potassium cyanate (2 mM), sodium bicarbonate (3 mM), and enzyme in 0.05 M potassium phosphate buffer, pH 7.6, in a volume of 2 mL. The amount of ammonia formed was determined after 10 min at 26 °C. Ammonia concentrations

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[1] Y. Sung, P. M. Anderson, and J. A. Fuchs, unpublished results.

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were determined as previously described (Anderson & Little, 1986).

Binding studies were carried out by equilibrium dialysis at 26 °C using dialysis cells and membranes as previously described (Anderson, 1977). The capacity of each chamber of the dialysis cells was 100 μL. The binding data were plotted and analyzed according to the Scatchard equation (Scatchard, 1949). Alternatively, in some cases, the data are plotted as r (moles of ligand bound per mole of enzyme) vs. total initial ligand concentration (concentration of ligand in each chamber of the dialysis cell if no binding occurred). All straight-line plots for the binding data, as well as the kinetic data, were treated by the method of least squares to obtain the best fit to the data. A subunit molecular weight of 17 008[2] was used for determining moles of ligand bound per mole of enzyme. Protein concentration was determined by measurement of the absorbance at 280 nm (Anderson, 1980).

[^{14}C]Malonate and H^{36}Cl were obtained from New England Nuclear Corp. and [^{14}C]oxalate and $Na_2{}^{14}CO_3$ were obtained from Research Products, International. [^{14}C]Malonate was purified by passage through a small column containing a cation-exchange resin in the hydrogen form. The radiolabeled purity of [^{14}C]malonate and [^{14}C]oxalate was determined by several thin-layer chromatographic systems, and the concentration of each was determined by titration and by inhibition of cyanase (compared with known concentrations of oxalate or malonate). The concentration of H^{36}Cl was verified by titration and by colorimetric determination of Cl^- (West & Coll, 1956).

Inhibition by dianions was determined by measuring the formation of ammonia with time in the presence of different concentrations of inhibitor in reaction mixtures containing 0.3 mM KOCN and 0.3 mM $NaHCO_3$ in 0.05 M potassium phosphate buffer, pH 6.8. At various times (which depended upon the inhibitor) after the reaction was initiated, the ammonia formed in a 2-mL volume removed from the reaction mixture was determined as described above. When slow-binding inhibition was observed (i.e., inhibition increased with time), the reaction was continued until a steady-state rate was achieved. For each inhibitor, the ratio v_0/v (v_0 = steady-state rate of the control; v = steady-state rate at a given inhibitor concentration) was determined as a function of inhibitor concentration. This ratio is linearly related to inhibitor concentration (Laidler, 1958), and this relationship was used to determine the concentration of inhibitor (dianion) that was required to give 50% inhibition under these assay conditions. Dianions were obtained from Sigma Chemical Co. or from Aldrich Chemical Co., Inc.

The rate of inhibition of cyanase (i.e., the increase in inhibition of cyanase with time) by oxalate, a slow-binding inhibitor, was determined by assaying 0.1-mL aliquots removed at various times after addition of oxalate from 2-mL reaction mixtures containing cyanase, oxalate, and other components as indicated in the text at 26 °C. Standard assay conditions were employed for measuring activity in the 0.1-mL aliquots, except the assay time was 5 min.

Results

Inhibition by Dianions. The effect of different dianions and other related anions on cyanase activity, expressed as the concentration of dianion required to give 50% inhibition of the steady-state rate under the described assay conditions, is summarized in Table I. Slow-binding inhibition was observed for all dianions in Table I except maleate, sulfite, fumarate, glutarate, and phosphonoacetate. For the dianions that exhibited the property of slow-binding inhibition, the time required to reach the reduced steady-state rate of activity varied from more than 40 min for oxalate and oxalacetate and 10–15 min for hydroxymalonate and sulfoacetate to less than 5 min for the others. Phthalate, citrate, isocitrate, aspartate, phosphate, pyrophosphate, tripolyphosphate, trimetaphosphate, 1,1-cyclobutanedicarboxylic acid, 1,2-cyclohexanedicarboxylic acid, and 1,2-cyclopentanedicarboxylic acid gave no observable inhibition.

Table I: Inhibition of Cyanase by Various Dianions

dianion	concn of dianion that gives 50% inhibition[a] (mM)	dianion	concn of dianion that gives 50% inhibition[a] (mM)
oxalate	0.004	methylmalonate	1.3
oxalacetate	0.006	3-nitropropionate	3.0
malonate	0.015	D-malate	3.1
hydroxymalonate	0.06	sulfate	3.3
maleate	0.07	succinate	4.0
sulfite	0.12	α-ketoglutarate	15.0
sulfoacetate	0.12	glutarate	43.0
fumarate	0.58	EDTA	64.0
L-malate	0.60	phosphonoacetate	100
thiosulfate	1.0		

[a] Assay conditions are described under Materials and Methods.

Scheme I

$$E + I \overset{K_I}{\rightleftharpoons} EI \xrightarrow{k_1} E\text{-}I$$

The concentration of sulfite required for 50% inhibition at pH 8.0 was found to be 4 times that required for 50% inhibition at pH 6.8 (Table I), whereas the concentration of the monoanions azide and acetate required for 50% inhibition at pH 8.0 was only twice that required for 50% inhibition at pH 6.8. This would suggest that inhibition by sulfite is probably due to the presence of monovalent bisulfite (the pK_a of bisulfite is 6.9 so that near-equivalent concentrations of bisulfite and sulfite are present at pH 6.8), which would have a reduced concentration relative to sulfite at pH 8.0. Kinetic experiments showed that inhibition by sulfite is competitive with respect to bicarbonate (cyanate saturating or nonsaturating) and noncompetitive with respect to cyanate (bicarbonate nonsaturating), which is consistent with inhibition by bisulfite as an analogue of bicarbonate.

As indicated above, oxalate exhibits slow-binding inhibition, and the time required to reach the reduced steady-state rate of cyanase activity is at least 40 min. This inhibition is reversible, but the dissociation of oxalate and the return of activity occur at a rate which is considerably slower than the slow rate of inhibition. Consequently, the decrease in activity of cyanase incubated with oxalate at different concentrations for various periods of time can be determined as described under Materials and Methods by simply adding a small aliquot to a standard assay mixture and measuring the ammonia formed after a short incubation period, during which time little reactivation of inhibited enzyme is observed and no further inhibition occurs. Two possibilities for the slow-binding inhibition by oxalate can be envisaged, as described by Morrison (1982). Oxalate could form a complex with enzyme in a rapid initial step followed by a slow isomerization step leading to a complex with a low dissociation constant (Scheme I where I represents oxalate). Since the second step can be considered essentially irreversible under the conditions of the experiments

[2] This value of the calculated subunit molecular weight based upon the amino acid sequence is a correction of the previously quoted value of 16 350 (Chin et al., 1983).

Scheme II

$$E + I \xrightarrow{k_1} E\text{-}I$$

Scheme III

$$\begin{array}{l} E + I \xrightarrow{k_1} E\text{-}I \\ + \\ A \\ \Big\Vert\!\!\downarrow K_A \\ EA \end{array}$$

described here as discussed above, this mechanism is analogous to that of an active-site-directed irreversible inhibitor, and the rate of inhibition would be described by eq 1 (Kitz & Wilson,

$$v = \frac{-d[E_t]}{dt} = \frac{k_1[I][E_t]}{K_I + [I]} = k_{inact}[E_t] \qquad (1)$$

1962) where $[E_t]$ = the concentration of active cyanase and $k_{inact} = k_1[I]/(K_I + [I])$. The value of k_{inact} would be dependent upon [I], and a plot of $1/k_{inact}$ vs. $1/[I]$ would give a straight line (slope = K_I/k_1) with an intercept on the $1/k_{inact}$ axis (intercept = $1/k_1$). Alternatively, oxalate could interact in an initial slow step directly with cyanase, leading to a complex with a low dissociation constant (Scheme II). In this case, the rate of inhibition would be given by eq 2, where k_{inact}

$$v = -d[E_t]/dt = k_1[I][E_t] = k_{inact}[E_t] \qquad (2)$$

$= k_1[I]$. A plot of $1/k_{inact}$ vs. $1/[I]$ would give a straight line with an intercept at the origin and slope = $1/k_1$.

As shown in Figure 1, inhibition by oxalate at pH 7.3 follows pseudo-first-order kinetics, characteristic of both schemes described above. However, the double-reciprocal plot of $1/k_{inact}$ (determined from the slopes in Figure 1) vs. $1/[I]$ gives a straight line with an intercept apparently at the origin (Figure 1, inset). Similar results are obtained at pH 6.8 and 5.7. These results would appear to be consistent with Scheme II, but not with Scheme I. The value of the apparent second-order rate constant, k_1, determined from the slope of the double-reciprocal plots increases with decreasing pH; the values for k_1 at pH 7.3, 6.8, and 5.8 are 45 ± 7, 130 ± 5, and 3040 ± 430 M^{-1} s^{-1}, respectively. The experiment described in Figure 1 carried out at pH 7.3 was repeated at 10 °C in order to follow the rate of inhibition at a higher concentration of oxalate (up to 3.2 mM). The same results were obtained (intercept apparently at the origin). However, as shown in Figure 1 (inset), at pH 7.7 the double-reciprocal plot is consistent with complex formation according to Scheme I; the values of K_I and k_1 (first-order rate constant) determined from these data are 7.8 mM and 6.7 min^{-1}, respectively.

The presence of either azide (analogue of cyanate) or bicarbonate at relatively low concentrations significantly reduces the rate of inactivation by oxalate. When the concentration of oxalate was 11 μM, the concentrations of azide and bicarbonate required to reduce k_{inact} by 50% were 0.015 and 0.5 mM, respectively; the k_{inact} was reduced by 90% when the concentrations of azide and bicarbonate were 0.2 and 4 mM, respectively. Binding of these ligands apparently precludes interaction with oxalate, which can be illustrated by Scheme III where K_A is the dissociation constant for the enzyme–ligand complex and A = azide or bicarbonate. The rate of inactivation according to Scheme III would be described by eq 3

$$v = \frac{k_1[I][E_t]}{1 + [A]/K_A} = k_1'[I][E_t] = k_{inact}[E_t] \qquad (3)$$

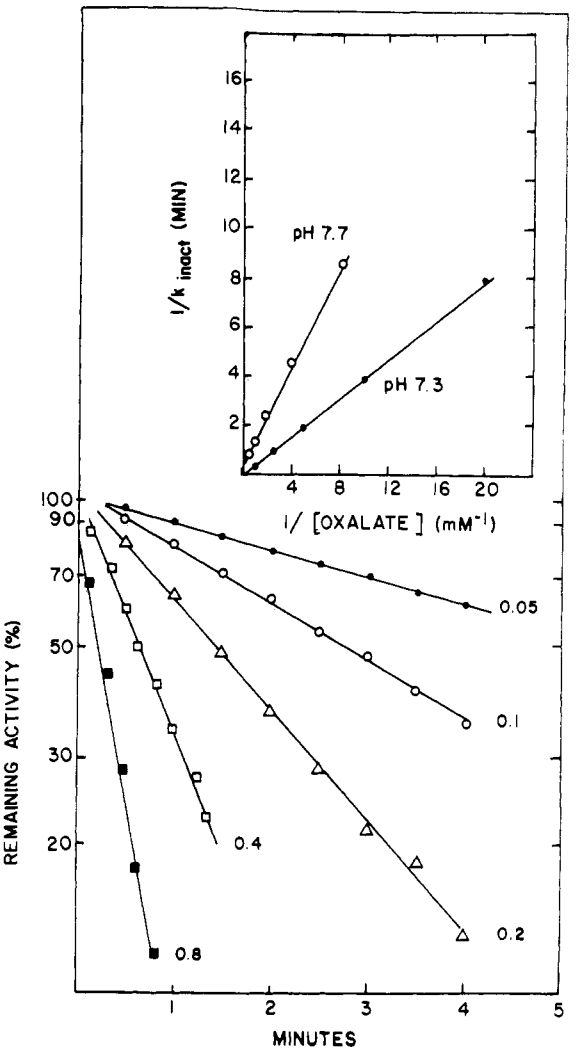

FIGURE 1: First-order plot of inhibition of cyanase by different concentrations of oxalate. The reaction mixtures contained oxalate as indicated (in millimolar) and cyanase (5 μg) in 2 mL of 0.05 M potassium phosphate buffer, pH 7.3 at 26 °C. At the indicated times, a 0.1-mL aliquot was removed and the enzyme activity determined as described under Materials and Methods. Inset: Double-reciprocal plots of k_{inact} vs. oxalate concentration. The values for k_{inact} at pH 7.3 were obtained from the slopes of the first-order plots. The values for k_{inact} at pH 7.7 were obtained in the same way except the reaction mixtures contained 0.1 M *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid buffer, pH 7.7, instead of potassium phosphate.

where $k_{inact} = k_1[I]$ and $k_1' = k_1/(1 + [A]/K_A)$. Thus, pseudo-first-order kinetics would be observed at fixed concentrations of I and A, and k_{inact} could be determined as described above. Plots of $1/k_{inact}$ vs. $1/[I]$ at fixed concentrations of A would give a straight line with an intercept at the origin, also as outlined above (Figure 1), from which k_1 could be obtained. A plot of [A] vs. $1/k_1$ should then give a straight line in which the intercept on the [A] axis = $-K_A$. Plots of $1/k_1$ vs. azide or bicarbonate concentration in which k_1 was determined as described above at different fixed concentrations of azide or bicarbonate were found to be linear and gave values of 0.01 and 0.6 mM, respectively, for the dissociation constants.

Equilibrium Dialysis Binding Studies. Scatchard plots of the binding of [^{14}C]oxalate to cyanase are shown in Figure 2. Binding is characterized by a low dissociation constant that decreases with decreasing pH. The dissociation constants calculated from these data at pH 7.8, 7.3, and 6.6 are 9, 3, and 0.4 μM, respectively. Of particular interest is the observation that the number of binding sites appear to be 0.5

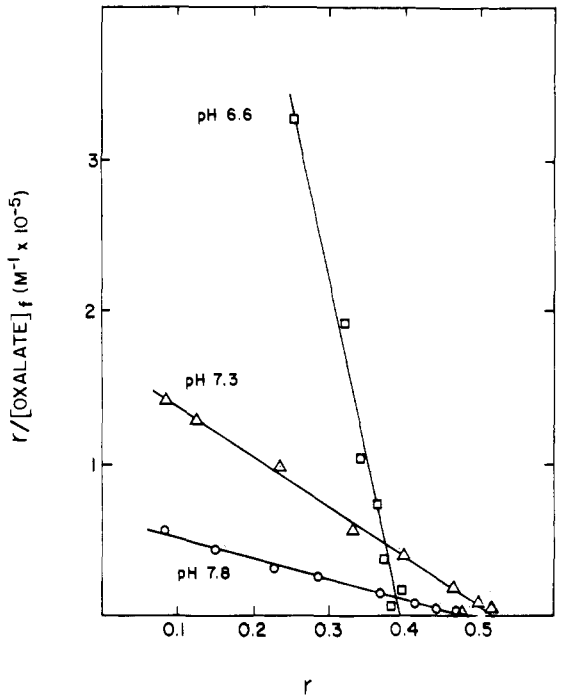

FIGURE 2: Scatchard plot of [^{14}C]oxalate binding as a function of pH. The range of [^{14}C]oxalate concentrations was 1–150 μM. The enzyme concentration in the chamber containing enzyme was 30 μM. The solutions also contained 0.5 mM EDTA and 0.05 M potassium phosphate at pH 6.6, 7.3, or 7.8, as indicated.

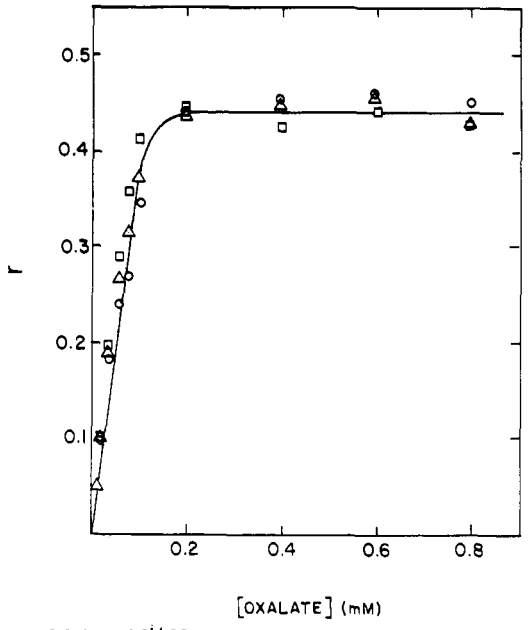

FIGURE 3: Moles of [^{14}C]oxalate bound per mole of cyanase (r) as a function of [^{14}C]oxalate concentration and pH. The enzyme concentration in the chamber containing enzyme was 400 μM. The solutions also contained 0.5 mM EDTA and 0.05 M potassium phosphate at pH 6.6 (□), 7.3 (△), or 7.8 (○) and [^{14}C]oxalate (initial concentration) as indicated.

per monomeric subunit (i.e., one oxalate bound per two subunits). The average values (means ± standard deviation) obtained from three or more different binding experiments at each pH as described in Figure 2 were 0.44 ± 0.01, 0.48 ± 0.02, and 0.47 ± 0.02 at pH 6.6, 7.3, and 7.8, respectively. The value of about 0.5 for moles of [^{14}C]oxalate bound per mole of monomeric subunit at pH 7.3 when [^{14}C]oxalate is saturating was obtained with cyanase under a number of different conditions, including (1) cyanase isolated from different extracts of *Escherichia coli* in which the gene had been cloned (1000-fold increase in cyanase activity over that in wild-type *Escherichia coli*), (2) cyanase isolated from wild-type *Escherichia coli* after induction by cyanate, (3) different concentrations of cyanase, and (4) cyanase that had been denatured by treatment with 6 M guanidine hydrochloride and then renatured by dialysis to remove the guanidine hydrochloride [denaturation conditions: 6 M guanidine hydrochloride, 0.6 mg of cyanase/mL, 0.05 M potassium phosphate buffer, pH 7.3, 3 mM dithiothreitol, and 0.5 mM ethylenediaminetetraacetic acid (EDTA), 20 min at 26 °C; dialysis buffer: 0.4 M potassium phosphate buffer, pH 7.3, 0.02 M sodium bicarbonate, and 1 mM dithiothreitol, 26 °C (denaturation under these conditions results in dissociation of subunits, loss of secondary structure, and loss of enzyme activity; dialysis under these conditions results in restoration of oligomeric structure and >85% of the catalytic activity)[3]].

Because of the high affinity for oxalate, the experiments in Figure 2 were carried out at relatively low cyanase concentration and low concentrations of [^{14}C]oxalate. In order to verify these results and to determine if an additional low-affinity binding site might be present that could be detected at a higher concentration of oxalate, binding studies were also carried out using much higher enzyme concentration. As shown in Figure 3, under these conditions, particularly at low pH, binding is essentially equivalent to a titration of the binding sites, and no additional binding occurs when the oxalate concentration exceeds half the concentration of cyanase monomeric subunits (up to 0.7 mM free oxalate). Monoanions and other dianions that are effective inhibitors of cyanase activity are also effective inhibitors of oxalate binding. When the concentration of [^{14}C]oxalate is 11 μM (pH 7.3, other conditions as described in Figure 2; r = 0.35), the concentrations of oxalacetate, azide, malonate, bicarbonate, and chloride required to reduce binding of [^{14}C]oxalate by 50% are 27, 110, 180, 3000, and 3100 μM, respectively.

The binding of [^{14}C]malonate resulted in Scatchard plots similar to those observed for [^{14}C]oxalate binding. In some experiments, the Scatchard plots appeared to be nonlinear (concave upward at very low concentrations of malonate), suggesting the possibility of negative cooperativity. The dissociation constant for malonate binding at pH 7.3 was found to be ≈50 μM. The value determined for the number of binding sites per monomer (r) was about 0.4 under a variety of different enzyme concentrations.

Scatchard plots of the binding of $^{36}Cl^-$ and [^{14}C]bicarbonate are shown in Figure 4. The dissociation constant for Cl^- under these conditions is 0.4 mM, and, like [^{14}C]oxalate binding, the value of r is approximately 0.5. When the concentration of $^{36}Cl^-$ is 0.8 mM (r = 0.38), the concentrations of azide and bicarbonate required to reduce $^{36}Cl^-$ binding by 50% are 80 and 9400 μM, respectively.

The dissociation constant for [^{14}C]bicarbonate is 0.24 mM. The presence of 0.23 mM oxalate essentially eliminates binding of [^{14}C]bicarbonate, while azide does not significantly affect [^{14}C]bicarbonate binding, even at concentrations as high as 10 mM. Relatively high concentrations of Cl^- are also required to reduce [^{14}C]bicarbonate binding; for example, only a small effect is observed when the Cl^- concentration is 2.5 mM. The latter observation together with the fact that very high concentrations of bicarbonate are required to significantly reduce $^{36}Cl^-$ binding suggests that Cl^- and bicarbonate can bind simultaneously, with Cl^- presumably binding at the cyanate binding site. Binding studies with $^{36}Cl^-$ and [^{14}C]bicarbonate

[3] R. M. Little and P. M. Anderson, unpublished results.

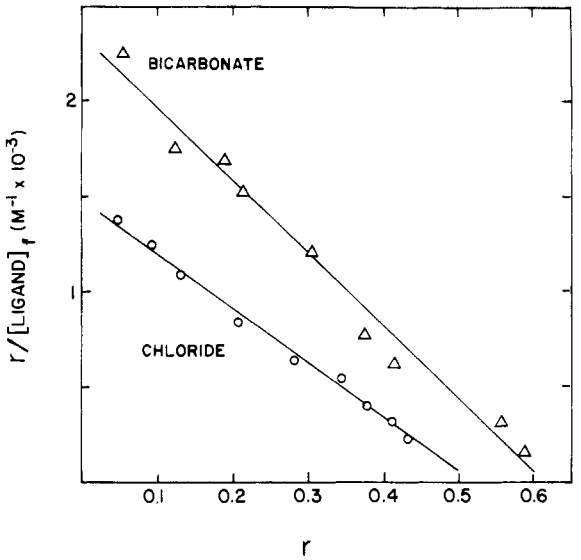

FIGURE 4: Scatchard plot of $^{36}Cl^-$ and [^{14}C]bicarbonate binding. The range of $^{36}Cl^-$ (○) and [^{14}C]bicarbonate (△) concentrations was 40–2000 and 40–4000 μM, respectively. The enzyme concentration in the chamber containing enzyme was 150 μM ($^{36}Cl^-$ binding) and 450 μM ([^{14}C]bicarbonate binding). The solutions also contained 0.5 mM EDTA and 0.05 M potassium phosphate, pH 7.3.

in the presence of added bicarbonate and Cl^-, respectively, showed that this is in fact the case; for example, the value of r for $^{36}Cl^-$ at 2.5 mM $^{36}Cl^-$ in the presence of 3 mM bicarbonate is 0.34, and the value of r for [^{14}C]bicarbonate at 3 mM [^{14}C]bicarbonate in the presence of 2.5 mM Cl^- is 0.54, giving a combined value for r of 0.88.

Discussion

The results of these studies are consistent with a mechanism of inhibition of cyanase by dianions that involves interaction with adjacent cyanate and bicarbonate substrate binding sites. This interaction is characterized by a significant degree of structural and/or isomeric (geometric and stereo) specificity; for example, the relative apparent affinities for succinate, methylmalonate, fumarate, and D-malate are considerably less than those for oxalacetate, malonate, maleate, and L-malate, respectively. The observed high affinity of cyanase for compounds such as oxalate, oxalacetate, malonate, and maleate apparently arises from their common structural feature as a *dianion*, since considerably higher concentrations of related monoanions such as acetate or formate are required to achieve significant inhibition (Anderson & Little, 1986). The high degree of specificity exhibited by dianions, the much higher affinity of several dianions compared to related small organic anions, and the slow-binding phenomenon exhibited by most dianions (but which varies considerably in magnitude from one dianion to another) would appear to reflect specific structural restraints for accommodation of a dianion within the cyanate and bicarbonate binding sites.

The kinetics of the slow-binding inhibition of cyanase by oxalate at pH 7.7 are consistent with Scheme I (initial complex formation followed by isomerization leading to a complex with a low dissociation constant). The K_I (≈7 mM) for initial complex formation is relatively high, much higher than the overall dissociation constant (≈0.009 mM) obtained from binding studies. Morrison (1982) has pointed out that if the value of the dissociation constant for initial complex formation in Scheme I (K_I) is much higher than the overall dissociation constant and the inhibitor concentration is varied in the region of the overall dissociation constant (i.e., K_I is high relative to the varied inhibitor concentrations), then Scheme I cannot be distinguished from Scheme II (Williams & Morrison, 1979). It seems likely that this is the explanation for why the kinetic studies at pH 7.3 and lower appear to be consistent with Scheme I rather than Scheme II (the sharply increased rates of inhibition of cyanase by oxalate at lower pH values necessitated use of oxalate at very low concentrations). The apparent second-order rate constant obtained by applying eq 2 (Scheme II) in these cases would then actually be comprised of the first-order rate constant divided by K_I (eq 1, Scheme I, where $K_I > [I]$).

The observation that the rate of inhibition of cyanase by oxalate is significantly decreased by relatively low concentrations of either azide (an analogue of cyanate) or bicarbonate and that these compounds have proportionately similar effects (at higher concentration) on equilibrium binding of oxalate to cyanase provides additional evidence that formation of the complex with the low dissociation constant involves both substrate sites. The observation that the overall dissociation constant for oxalate is lower than the apparent dissociation constants for either bicarbonate or cyanate (Anderson & Little, 1986) is also consistent with binding of dianions involving both substrate sites.

Other dianions and chloride also effectively compete with oxalate for binding. Azide and chloride appear to bind primarily at the cyanate site, since (1) very high concentrations of bicarbonate are required to compete with chloride binding, (2) very high concentrations of azide, in particular, and also chloride are required to effect a significant decrease in bicarbonate binding, (3) low concentrations of azide effectively reduce chloride binding, and (4) chloride and bicarbonate can both bind under conditions where the binding of each is near maximal. In contrast, oxalate and other dianions inhibit binding of both bicarbonate and chloride. The observation that bicarbonate and analogues of cyanate (chloride, azide) bind at different sites and can bind simultaneously as well as independently supports previous initial velocity kinetic studies that provided evidence favoring a rapid equilibrium random mechanism in which bicarbonate acts as a substrate and cyanate and bicarbonate bind at adjacent anion binding sites (Anderson & Little, 1986).

The discovery that the stoichiometry of oxalate binding at saturation per subunit appears to be 0.5 is somewhat surprising. This same stoichiometry is observed for malonate and for bicarbonate and chloride. The possibility that the extinction coefficient at A_{280} utilized to measure cyanase concentration is in error and accounts for the unusual stoichiometry is unlikely, since the value utilized is in close agreement with dry weight determinations and other stoichiometric procedures employed to confirm the value.[3] The possibility that the stoichiometry is an artifact due, for example, to partial inactivation during isolation, resulting in elimination of half the binding sites or irreversible binding of an unknown inhibitor at half the binding sites during isolation, cannot be excluded. However, the consistency of results obtained using cyanase of various origin (e.g., cloned, wild type, or renatured) supports the view that the observed stoichiometry is due to "apparent half-site binding". The exact oligomeric structure of cyanase has not yet been elucidated (the enzyme has been crystallized,[3] and preliminary X-ray analysis is in progress[4]), but the quaternary structure of the enzyme is a prominent and somewhat unusual feature of the enzyme. The reaction catalyzed is relatively simple in terms of what is to be accomplished, which

[4] R. B. Honzatko and P. M. Anderson, unpublished results.

is effectively hydration of cyanate to give carbamate (Anderson, 1980). There is no kinetic evidence for positive or negative cooperativity that might be mediated through subunit interactions of an oligomeric structure (Anderson & Little, 1986). Although the biological role of cyanase is unknown, it is difficult to envisage the need for extensive quaternary structure related to a metabolic regulatory role involving an induced enzyme that catalyzes decomposition of cyanate. The oligomeric structure is very stable, and there is no evidence that the enzyme undergoes dissociation under normal conditions (Anderson, 1980). However, preliminary studies in our laboratory indicate that reversible dissociation to inactive dimers can be demonstrated by covalent modification reactions, suggesting that the oligomeric structure is required for catalytic activity.[3] The involvement of bicarbonate as a substrate in this reaction and the observation of apparent half-site binding may be related to the oligomeric structure, perhaps involving intersubunit binding sites.

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Substrate Activity of Synthetic Formyl Phosphate in the Reaction Catalyzed by Formyltetrahydrofolate Synthetase†

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ABSTRACT: Formyl phosphate, a putative enzyme-bound intermediate in the reaction catalyzed by formyltetrahydrofolate synthetase (EC 6.3.4.3), was synthesized from formyl fluoride and inorganic phosphate [Jaenicke, L. v., & Koch, J. (1963) *Justus Liebigs Ann. Chem. 663*, 50–58], and the product was characterized by ^{31}P, ^{1}H, and ^{13}C nuclear magnetic resonance (NMR). Measurement of hydrolysis rates by ^{31}P NMR indicates that formyl phosphate is particularly labile, with a half-life of 48 min in a buffered neutral solution at 20 °C. At pH 7, hydrolysis occurs with P–O bond cleavage, as demonstrated by ^{18}O incorporation from $H_2{}^{18}O$ into P_i, while at pH 1 and pH 13 hydrolysis occurs with C–O bond cleavage. The substrate activity of formyl phosphate was tested in the reaction catalyzed by formyltetrahydrofolate synthetase isolated from *Clostridium cylindrosporum*. Formyl phosphate supports the reaction in both the forward and reverse directions. Thus, N^{10}-formyltetrahydrofolate is produced from tetrahydrofolate and formyl phosphate in a reaction mixture that contains enzyme, Mg(II), and ADP, and ATP is produced from formyl phosphate and ADP with enzyme, Mg(II), and tetrahydrofolate present. The requirements for ADP and for tetrahydrofolate as cofactors in these reactions are consistent with previous steady-state kinetic and isotope exchange studies, which demonstrated that all substrate subsites must be occupied prior to catalysis. The k_{cat} values for both the forward and reverse directions, with formyl phosphate as the substrate, are much lower than those for the normal forward and reverse reactions. Kinetic analysis of the formyl phosphate supported reactions indicates that the low steady-state rates observed for the synthetic intermediate are most likely due to the sequential nature of the normal reaction.

Chemical activation of substrates by a nucleoside triphosphate dependent phosphorylation is believed to be an essential step in the mechanisms of several ligase enzymes, including that of N^{10}-formyltetrahydrofolate (N^{10}-formyl-H_4folate)[1] synthetase (EC 6.3.4.3) (Himes & Harmony, 1973). This enzyme catalyzes an ATP-dependent formylation of H_4-folate into N^{10}-formyl-H_4folate:

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[1] Abbreviations: H_4folate, tetrahydrofolate; DSS, 3-(trimethylsilyl)-1-propanesulfonic acid (sodium salt); ADPβS, adenosine 5′-*O*-(2-thiodiphosphate); Ap_5A, P^1,P^5-di(adenosine-5′) pentaphosphate; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; NMR, nuclear magnetic resonance; TMS, tetramethylsilane; HPLC, high-performance liquid chromatography; Tris, tris(hydroxymethyl)aminomethane.

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