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# Inhibition of Isocitrate Lyase by 3-Nitropropionate, a Reaction-Intermediate Analogue<sup>†</sup>

John V. Schloss<sup>‡</sup> and W. W. Cleland\*

**ABSTRACT:** In the back-reaction of isocitrate lyase, condensation of succinate with glyoxylate, the probable mechanism involves formation of a carbanion in the  $\alpha$  position of succinate. 3-Nitropropionate (when fully ionized) should thus be a potent inhibitor of the enzyme as an analogue of the carbanion intermediate. At pH 8, 3-nitropropionate (apparent  $K_i \leq 17$  nM) binds 65 000 times more tightly to isocitrate lyase from *Pseudomonas indigofera* than succinate ( $K_m = 1.1$  mM), and the  $K_i$  value for the fully ionized form is  $\leq 1.5$  nM. Nitropropionate exhibits slow-binding inhibition of the enzyme, with an association rate at pH 8 of  $1050 \text{ M}^{-1} \text{ s}^{-1}$  (at low nitropropionate) and a dissociation rate of  $\leq 0.0011 \text{ min}^{-1}$ . Nitropropionate also appears to bind to isocitrate lyase in a two-step fashion, forming an initial, rapid complex with an apparent  $K_i$  of 0.25 mM at pH 8 (23  $\mu\text{M}$  for the fully ionized form), followed by conversion to a tighter ( $\geq 14$  000-fold) complex at a maximal rate of  $16 \text{ min}^{-1}$ . Itaconate and maleate ( $\text{sp}^2$  at the  $\alpha$  position) can also be considered analogues of the

carbanion of succinate ( $\text{sp}^2$  at the  $\alpha$  position in its aci form). These inhibitors also bind more tightly than succinate (260- and 6-fold, respectively) and exhibit slow-binding inhibition (dissociation rate = 0.3 and  $4 \text{ min}^{-1}$ , respectively).  $\text{Mg}^{2+}$  is required for tight binding of nitropropionate. Glyoxylate stimulates and succinate prevents binding of nitropropionate. Glyoxylate binds to enzyme-nitropropionate considerably tighter than to free enzyme as judged by gel filtration with [ $^{14}\text{C}$ ]glyoxylate. Under single turnover conditions (excess enzyme), 1-hydroxy-2-nitro-1,3-[ $^{14}\text{C}$ ]propanedicarboxylate was converted to [ $^{14}\text{C}$ ]glyoxylate and presumably to nitropropionate, but the reverse reaction did not occur. With excess 1-hydroxy-2-nitro-1,3-propanedicarboxylate, isocitrate lyase converted 41% to glyoxylate when the latter was reduced to glycolate with lactate dehydrogenase. At pH 8 the racemic nitro analogue of isocitrate has a  $K_m$  of 95  $\mu\text{M}$  and  $V_{\max}$  0.8% that of  $D_s$ -isocitrate ( $K_m = 85 \mu\text{M}$ ).

The glyoxylate cycle is an essential metabolic pathway for gluconeogenesis from fatty acids. Isocitrate lyase (EC 4.1.3.1) and malate synthase (EC 4.1.3.2) are the only enzymes unique to this metabolic pathway. These two enzymes are present during the germination of various seeds (Beevers, 1979) and spores (Gemmrich, 1979, 1980; DeMaggio et al., 1979), during the development of nematode larvae (Khan & McFadden, 1980; McKinley & Trelease, 1978; Patel & McFadden, 1978a), and during growth of various microorganisms (Kornberg & Elsdén, 1961). The relative importance of the glyoxylate cycle in seed germination (Khan & McFadden, 1979) and nematode development (Patel & McFadden, 1978b) has been demonstrated by their respective sensitivity to itaconate (2-methylenesuccinate), a potent inhibitor of isocitrate lyase (Rao & McFadden, 1965; Williams et al., 1971; Rittenhouse & McFadden, 1974). Metabolic specificity of itaconate for isocitrate lyase is indicated by its inhibition of the growth of *Pseudomonas indigofera* (McFadden & Purohit, 1977) and other procaryotes (Bellion & Kelley, 1979) only under conditions which required gluconeogenesis from one- or two-carbon substrates.

Inhibition of isocitrate lyase by itaconate represents something of an anomaly.  $\alpha$ -Methylsuccinate is a very poor inhibitor of the enzyme (Rao & McFadden, 1965) so the  $\alpha$ -methylene group of itaconate is not likely to contribute to binding, yet itaconate binds considerably tighter (125 $\times$  at pH 7.7) than the substrate succinate (Williams et al., 1971).

Recently, nitro analogues of the substrates of several lyases [fumarase and aspartase (Porter & Bright, 1980), aconitase (Schloss et al., 1980), and enolase (Anderson, 1981a)] have been found to bind to these enzymes with an affinity greater than the analogous substrate. Potent inhibition was due to the ionized (presumably aci) form of these carbon acids (nitroalkanes generally having pK values from 9 to 11). In the aci form of nitroalkanes, electrons have been delocalized from the carbanion to the oxygens of the adjacent nitro group to yield  $\text{sp}^2$  hybridization for the  $\alpha$  carbon (see Scheme I). Stabilization of the analogous form of the substrate carbanion may play a role in catalysis by various lyases. Thus inhibitors which are *trigonal* in the position  $\alpha$  to the carboxyl of lyase substrates may act as analogues of the carbanion intermediate and be very tightly bound. We considered itaconate to be such an inhibitor. As an extension of this proposal, we have examined isocitrate lyase for inhibition by 3-nitropropionate, a nitro analogue of succinate which can readily form a carbanion. We have also compared this inhibition to that by itaconate, maleate, and 3-phosphonopropionate.

## Experimental Procedures

### Materials

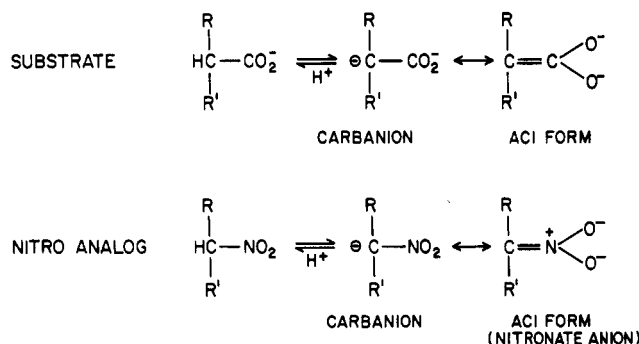
$D_s$ -Isocitrate, DL-isocitrate trisodium salt, TAPS,<sup>1</sup> glutathione, glyoxylic acid, isocitrate dehydrogenase (NADP specific), lactate dehydrogenase, NADP, NADH, itaconate, and Sephadex (G-25 and G-100) were purchased from Sigma Chemical Co. 3-Nitropropionic acid was a product of Aldrich Chemical Co. 3-Phosphonopropionic acid was obtained from Richmond Organics. 1-Hydroxy-2-nitro-1,3-propanedicarboxylate was synthesized by a published procedure (Schloss

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<sup>1</sup> Abbreviations: EDTA, (ethylenedinitrilo)tetraacetic acid; NMR, nuclear magnetic resonance; TAPS, 3-[[tris(hydroxymethyl)methyl]-amino]propanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane.

Scheme I



et al., 1980). *Pseudomonas indigofera* (ATCC 19706) was obtained from the American Type Culture Collection and maintained on YM broth (Difco 0711) and YM agar (Difco 0712). Isocitrate lyase was purified from *P. indigofera* by the method of Rittenhouse & McFadden (1974). The specific activity of the enzyme at 25 °C, pH 8, with D<sub>5</sub>-isocitrate in the assay described below was 13  $\mu\text{mol min}^{-1} \text{mg}^{-1}$ . This is 40% of the value reported at 30 °C under slightly different conditions by McFadden (1969). By the criterion of slab gel electrophoresis the enzyme was nearly homogeneous, although several minor contaminants could be detected at high protein loading.

### Methods

Elemental analyses were done by Galbraith Laboratories, Knoxville, TN. Melting points were obtained on a Fisher-Johns melting point apparatus and are uncorrected.

**Large Scale Culture of *P. indigofera*.** Initial attempts to grow *P. indigofera* on the 0.3% sodium butyrate medium described by McFadden (1969) were unsuccessful. Substitution of the inorganic components of the medium with those of Ormerod et al. (1961) resulted in vigorous and reliable growth on butyrate (in the absence of yeast extract). A 20-L butyrate-grown culture was used as inocula for a 300-L culture (initial OD<sub>650</sub> = 0.31). Temperature of the large scale culture was kept at 27 °C, and pH 7 was maintained by autotitration with butyric acid. Stirring and aeration rates were varied to achieve a dissolved O<sub>2</sub> concentration  $\geq 30\%$  of air saturation. The culture was harvested 54 h after inoculation (OD<sub>650</sub> = 18) by continuous flow centrifugation with a yield of 3.3 kg of cells (wet weight).

**Synthesis of [<sup>14</sup>C]Glyoxylate and 1-Hydroxy-2-nitro-1,3-[<sup>14</sup>C]propanedicarboxylate.** [<sup>14</sup>C]Oxalic acid from New England Nuclear was reduced electrolytically by the method of Krupka & Towers (1958). A small quantity of mercury (1 mL) containing a 24-gauge platinum wire protruding from the end of a 50- $\mu\text{L}$  capillary pipet was placed in the bottom of a shell vial, and 5.6 mg of [<sup>14</sup>C]oxalic acid (44  $\mu\text{mol}$ ; 250  $\mu\text{Ci}$ ) in 1 mL of 2% sulfuric acid (0.72 N) was layered above the mercury. A second platinum wire was placed just below the surface of the oxalic acid solution, and the two electrodes were connected to three 1.5-V batteries in series such that the mercury contained the cathode. After 5 min of reduction, the mixture contained 47  $\mu\text{mol}$  of glyoxylate (107%) as determined by assay with lactate dehydrogenase. To 0.9 mL of the reduced oxalic acid ( $3.4 \times 10^8$  cpm) were added 0.1 mL of 66 mg/mL nitropropionic acid (56  $\mu\text{mol}$ ) and 100 mg of sodium carbonate (0.94 mmol). After 30 min, 1 g of Dowex 50 (H<sup>+</sup> form) was added to stop the reaction, the slurry was filtered through a Pasteur pipet packed with glass wool, and the Dowex 50 was washed with four 0.25-mL portions of water. To the combined eluate were added 10  $\mu\text{L}$  of concentrated HCl and

0.6 g of NaCl. The reaction product was extracted (87% based on radioactivity) with 10 2-mL portions of diethyl ether. The ether extract was concentrated to approximately 1 mL with a stream of nitrogen and loaded onto a column (1.8  $\times$  25 cm) of dry SilicAR CC-4 silica gel powder (Mallinckrodt) which had not been pretreated in any way. The column was eluted with anhydrous ethyl ether, and the majority of radioactivity came off the column centered at 40 mL of eluant, coincident with 1-hydroxy-2-nitro-1,3-propanedicarboxylic acid and resolved from nitropropionic acid, which elutes slightly earlier. Fractions collected between 30 and 60 mL of eluant were pooled. The ether pool was evaporated to a thin film with nitrogen and redissolved in 5 mL of water. The pH of the solution was adjusted to 6 on ice by the dropwise addition of 0.1 M cyclohexylamine. On the basis of the recovery of radioactivity ( $1.85 \times 10^8$  cpm), a 54% overall yield was obtained. Examination of the product ( $R_f$  0.15) by ion-exchange paper chromatography (Whatman DE 81, eluted with 0.1 M Tris-HCl, pH 8) revealed the presence of a radiolabeled contaminant (7.5%) which was probably glyoxylate ( $R_f$  0.50). The presence of a small amount of glyoxylate (5.0%) in the product was confirmed by assay with lactate dehydrogenase. Glyoxylate's presence is presumably due to a small amount of decomposition of the condensation product during workup [see Schloss et al. (1980)]. Incubation of a small portion of the neutralized solution at room temperature overnight resulted in an increase in the glyoxylate contamination to 23% as judged by chromatographic analysis. The majority of sample (22  $\mu\text{mol}$ ,  $1.6 \times 10^8$  cpm) was frozen immediately after neutralization and lyophilized.

**Synthesis of  $\alpha$ -Methylisocitrate.**  $\alpha$ -Methyl-*cis*-aconitic anhydride was prepared by the method of Gawron & Mahajan (1966). The anhydride (250 mg, 1.47 mmol) was hydrolyzed with K<sub>2</sub>CO<sub>3</sub> (300 mg, 2.2 mmol) and incubated (50 mL volume) at pH 7 with 10 units of beef liver aconitase (Henson & Cleland, 1967) overnight. After the reaction mixture (pH  $\approx$  0.1) was acidified with concentrated HCl, the solution was saturated with NaCl and extracted 5 times with 100-mL portions of ether. The ether was allowed to evaporate, and the residue was dissolved in a small portion of water (10 mL) and then applied to a 1.8  $\times$  26 cm column of Dowex 1-X8-Cl<sup>-</sup>. Elution was conducted isocratically with 0.1 N HCl; 400-drop fractions were collected.  $\alpha$ -Methylisocitrate eluted at fraction 21 (18–25), followed by  $\alpha$ -methyl-*cis*-aconitate centered by fraction 40 (33–47). Fractions containing  $\alpha$ -methylisocitrate were pooled, rotary evaporated to dryness, resuspended in a small (5 mL) volume of water, and dried over P<sub>2</sub>O<sub>5</sub> and KOH pellets. The crystalline  $\alpha$ -methylisocitric acid  $\gamma$ -lactone recovered (100 mg, 0.53 mmol) had a melting point of 142–144 °C. The reported melting point for the racemic threo mixture is 183–186 °C (Beach et al., 1977). Anal. Calcd for C<sub>7</sub>H<sub>8</sub>O<sub>6</sub>: C, 44.69; H, 4.29. Found: C, 44.50; H, 4.26. Estimation of  $\alpha$ -methylisocitrate enzymatically with isocitrate lyase and lactate dehydrogenase after saponification of the  $\gamma$ -lactone gave 97% of theory. Beach et al. (1977) have reported methylcitrate not to be produced from  $\alpha$ -methyl-*cis*-aconitate by aconitase and synthetic methylcitrate not to be a substrate. Consistent with this observation, we could not detect methylcitrate in equilibrium mixtures produced from methyl-*cis*-aconitate and aconitase by 270-MHz NMR (data not shown).

**Assay of Isocitrate Lyase.** All enzyme assays were conducted at 25 °C and pH 8. Purified isocitrate lyase concentrations were determined from the absorbancy at 280 nm by assuming an  $E_{1\%}^{1\text{cm}}$  of 17.1 (McFadden, 1969). Each lyase assay contained 3.33 mM DL-isocitrate or 0.33 mM threo-

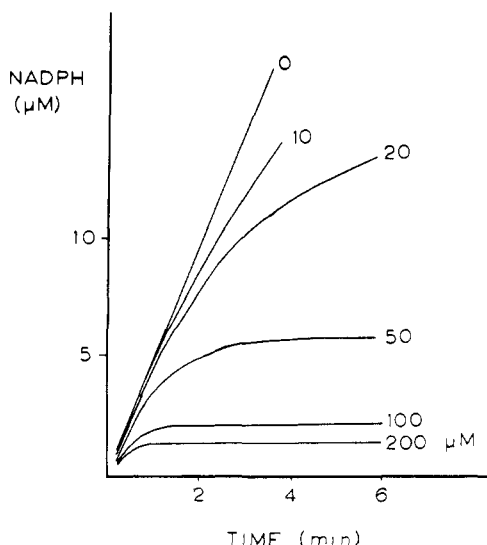


FIGURE 1: Assay progress curves in the presence of the micromolar concentrations of 3-nitropropionate designated in the figure. Nitropropionate was preequilibrated overnight at pH 8 prior to addition of 3.33 mM each of glyoxylate and succinate and initiation of the assay with enzyme. For the details of the coupled assay with isocitrate dehydrogenase, see Methods.

D<sub>5</sub>-α-methylisocitrate, 10 mM magnesium acetate, 0.17 mM NADH, 33 μg/mL (methylisocitrate assay) or 0.17 mg/mL (isocitrate assay) lactate dehydrogenase, and 0.1 M TAPS-KOH, pH 8.0. Each condensation assay contained 3.33 mM sodium succinate, 3.33 mM sodium glyoxylate, 10 mM magnesium acetate, 0.17 mM NADP, 0.15 unit/mL NADP-specific isocitrate dehydrogenase, and 0.1 M TAPS-KOH, pH 8.0. Prior to either assay the enzyme was preincubated with 5 mM glutathione, 1 mM EDTA, and 0.1 M TAPS-KOH, pH 8.0. The rates with the specified levels of isocitrate or methylisocitrate in the cleavage assays were 72% and 5.9%, respectively, of the rate seen in the condensation assay with the indicated levels of succinate and glyoxylate. *threo*-D<sub>5</sub>-Methylisocitrate was determined to have a  $K_m$  of 0.15 mM, which compares favorably with a previously determined value of 0.1 mM (McFadden et al., 1972).

## Results

**Inhibition by 3-Nitropropionate.** Assay of isocitrate lyase in the presence of 3-nitropropionate resulted in nonlinear time courses (Figure 1). The slow loss of activity in the presence of 3-nitropropionate was examined further by preincubating the enzyme (0.11 mg/mL, 0.53 μM) with 3-nitropropionate (5 μM, preequilibrated to pH 8), in 0.1 M TAPS-KOH, pH 8, 5 mM glutathione, 0.5 mM EDTA, and 5 mM magnesium acetate, or various levels of magnesium acetate as specified (Figure 2). Aliquots (10 μL) of the preincubation mixtures were removed after various times, and the initial rates obtained upon dilution into an assay mixture (3 mL) were determined. Isocitrate lyase lost activity in a pseudo-first-order fashion in the presence of 3-nitropropionate until less than 5% of the initial enzymic activity remained (Figure 2). Mg<sup>2+</sup> was required for the time-dependent loss of activity, as its exclusion from the preincubation mixtures dramatically retarded inhibition of isocitrate lyase by nitropropionate (Figure 2A). By contrast, succinate prevented the time-dependent inhibition of isocitrate lyase by nitropropionate (Figure 2B). The rate of inhibition was halved by 2 mM succinate, with 50 mM succinate giving virtually complete protection. Glyoxylate stimulated the rate of nitropropionate inhibition (Figure 2C). The rate of inhibition was increased 2.3-fold by including 500

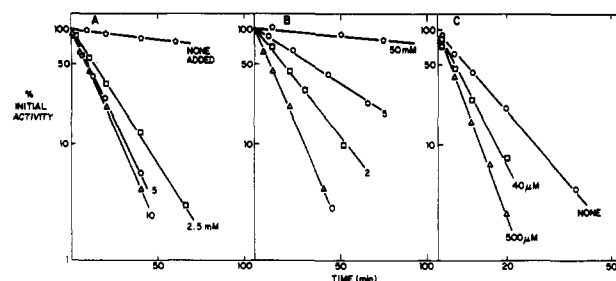


FIGURE 2: (A) Effect of Mg<sup>2+</sup> on nitropropionate binding. Enzyme was incubated with 0 (☆), 2.5 (□), 5 (○), or 10 mM (Δ) magnesium acetate in the presence of 5 μM 3-nitropropionate (preequilibrated to pH 8). (B) Effect of succinate on nitropropionate binding. The enzyme was incubated with 0 (Δ), 2 (□), 5 (○), or 50 mM (☆) sodium succinate in the presence of 5 μM 3-nitropropionate. (C) Effect of glyoxylate on nitropropionate binding. The enzyme was incubated with 0 (○), 40 (□), or 500 μM (Δ) sodium glyoxylate in the presence of 5 μM 3-nitropropionate. Aliquots (10 μL) of the preincubation mixtures were added at the indicated times to 3-mL assay mixtures containing 3.33 mM glyoxylate and succinate (isocitrate dehydrogenase coupled assay), and initial rates were measured.

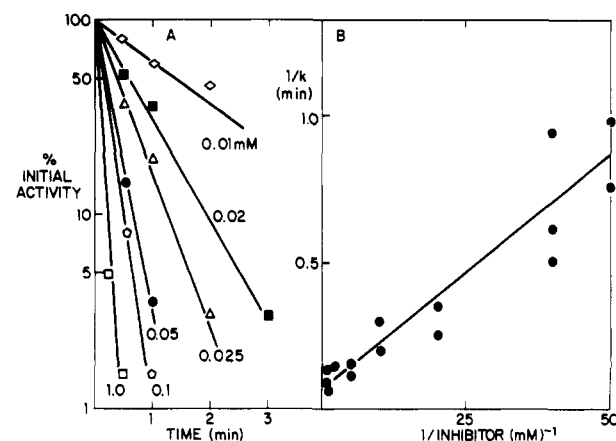


FIGURE 3: Determination of nitropropionate association rate. (A) The percent of initial enzymic activity remaining after various times is shown for 0.01 (◇), 0.02 (■), 0.025 (Δ), 0.05 (●), 0.1 (☆), and 1.0 mM (□) 3-nitropropionate (preequilibrated to pH 8) incubated with isocitrate lyase. The isocitrate coupled assay was used. (B) A replot of the data shown in (A) and of similar data. The reciprocal of the first-order rate constant is plotted vs. [inhibitor]<sup>-1</sup>.

μM glyoxylate in the preincubation mixture, with 40 μM glyoxylate giving approximately half of maximal stimulation. Higher concentrations of glyoxylate (>0.5 mM) retarded the rate of inhibition (data not shown). The dependence of the inhibition rate on nitropropionate concentration is shown in Figure 3. Preincubation was conducted at pH 8 in the presence of 0.5 mM glyoxylate and 5 mM magnesium acetate. At the highest preincubation concentration of nitropropionate, 1 mM, the rate of inhibition was extremely rapid, with only 5% of the initial enzymic activity remaining after 15 s (Figure 3A). A replot of these and similar data is shown in Figure 3B. Kinetic constants were obtained by fitting data to the equation  $k = k_{max}[I]/(K + [I])$  by the least-squares method (Cleland, 1967). The rate of inhibition appeared to saturate with a maximal value of  $15.8 \pm 2.7 \text{ min}^{-1}$ . The concentration of 3-nitropropionate giving a half-maximal rate of inhibition was  $0.25 \pm 0.12 \text{ mM}$ , and a second-order rate constant (rate of inhibition at low nitropropionate) of  $1050 \pm 370 \text{ M}^{-1} \text{ s}^{-1}$  was obtained at this pH.

Isocitrate lyase (0.11 mg/mL) was preincubated at pH 8 with 5 μM 3-nitropropionate until inhibition was complete to assess the reversibility of 3-nitropropionate inhibition; then the enzyme was diluted 3000-fold into an assay mixture at the

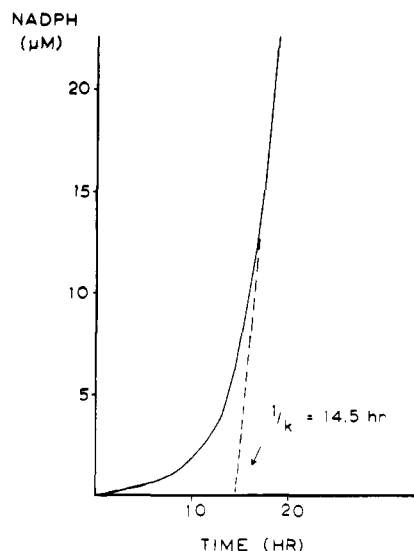


FIGURE 4: Reversal of nitropropionate inhibition of isocitrate lyase upon dilution into an assay mixture. Details are given in the text (isocitrate dehydrogenase coupled assay used with 3.33 mM glyoxylate and 16.7 mM succinate).

same pH containing 16.7 mM succinate. The time course for this assay is shown in Figure 4. Enzymic activity was regained slowly in an apparent first-order fashion and giving a final steady-state rate. The rate constant for the lag in this assay was  $0.0011 \text{ min}^{-1}$ . Since the final rate achieved was only about 25% of an uninhibited assay, the rate constant obtained is an overestimate of the dissociation rate constant. Enzyme incubated under similar conditions in the absence of nitropropionate lost no activity over this time period. This rate constant ( $\leq 0.0011 \text{ min}^{-1}$ ) together with the previously determined association rate constant ( $1050 \text{ M}^{-1} \text{ s}^{-1}$ ) gives a dissociation constant of  $\leq 17 \text{ nM}$  at pH 8.<sup>2</sup> Use was made of the slow rate of ionization of the carbon acid (Porter & Bright, 1980) to determine the effect of the ionization state of 3-nitropropionate on its rate of inhibition. Figure 5 illustrates the results of assays conducted in the presence of 3-nitropropionate (0.1 mM) in which the inhibitor was introduced initially as the free acid, preequilibrated to the assay pH of 8.0, or fully ionized (preincubated in 0.1 N KOH). Inhibition of the enzyme develops far more rapidly when nitropropionate is present initially fully ionized, although virtually complete inhibition was finally reached in each case. At pH 8 the carbon acid of 3-nitropropionate is only 9.1% ionized ( $\text{pK} = 9.0$ ; Porter & Bright, 1980). Consistent with its partial degree of ionization, 3-nitropropionate preequilibrated to the assay pH gave a rate of inhibition intermediate between that seen when either fully ionized or un-ionized carbon acid was present at the onset of the assay.

The time courses in Figure 5 were simulated by solving simultaneously the differential equations for the rates of change of (1) the nitronate form of 3-nitropropionate, (2) the tight enzyme-nitropropionate complex formed with a rate constant of  $16 \text{ min}^{-1}$ , and (3) the rate of production of NADPH in the assay, using the inhibition and rate constants determined in the present work. The curves in Figure 5 were very accurately reproduced if the  $K_m$  of succinate in the system was 1.83 mM

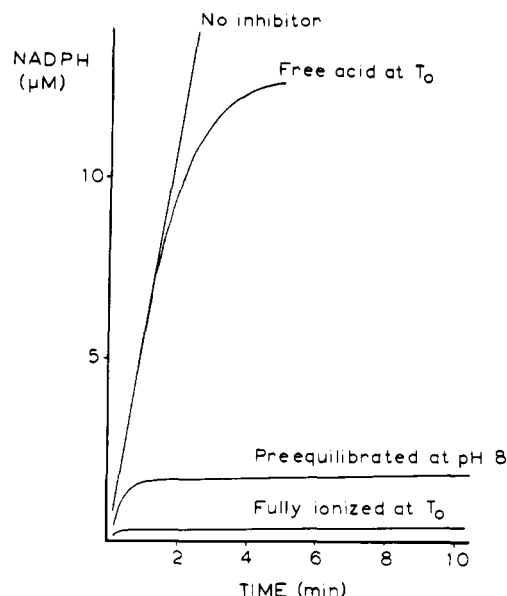


FIGURE 5: Effect of the ionization state of 3-nitropropionate on the rate of inhibition of isocitrate lyase. Assay progress curves are shown in which no inhibitor is present or 0.1 mM 3-nitropropionate was added initially as the free acid, preequilibrated in 0.1 N KOH, or preequilibrated to the assay pH. The isocitrate dehydrogenase coupled assay with 3.33 mM glyoxylate and succinate was used.

and if the rate constant for protonation of the carbanion of 3-nitropropionate was assumed to be the same at pH 8 as the value of  $0.1 \text{ min}^{-1}$  measured at pH 7 by Porter & Bright (1980) (this means that the reaction in this pH range is  $\text{R}^- + \text{H}_2\text{O} \rightleftharpoons \text{RH} + \text{OH}^-$ , rather than  $\text{R}^- + \text{H}^+ \rightleftharpoons \text{RH}$ ).

**Binding Studies with [ $^{14}\text{C}$ ]Glyoxylate.** The effect of nitropropionate bound to isocitrate lyase on the enzyme's affinity for glyoxylate was assessed by gel filtration with [ $^{14}\text{C}$ ]glyoxylate. Gel filtration experiments were conducted by using columns of both Sephadex G-25, with which partial resolution of enzyme and glyoxylate could be achieved rapidly (Figure 6A), and Sephadex G-100, with which a more complete separation of enzyme and glyoxylate was obtained, but requiring more time due to slower flow rates (Figure 6B,C). When enzyme (5.3  $\mu\text{M}$ ), nitropropionate (100  $\mu\text{M}$ ), and [ $^{14}\text{C}$ ]glyoxylate (4  $\mu\text{M}$ ) were passed rapidly through G-25 after a brief (15-min) preincubation, the majority of glyoxylate eluted in the position of the enzyme (Figure 6A). When the preincubation mixture was passed through G-100 to effect greater resolution of enzyme and glyoxylate, less glyoxylate eluted in the position of enzyme (Figure 6B). However, the remaining glyoxylate eluted in a slightly earlier position than free glyoxylate, and the peak was skewed, trailing into the enzyme-glyoxylate peak. This elution profile is consistent with all of the glyoxylate being bound to enzyme initially and being slowly released during chromatography. When nitropropionate was omitted from the preincubation mixture, some glyoxylate still eluted in the position of enzyme, but the majority eluted in a position identical with that obtained when glyoxylate was passed through the column in the absence of enzyme (Figure 6B). The enzyme-nitropropionate-[ $^{14}\text{C}$ ]glyoxylate preincubation mixture was spiked with a large excess of cold glyoxylate (10 mM) just prior to gel filtration to confirm that glyoxylate was released rapidly (relative to the rate of nitropropionate release). Excess unlabeled glyoxylate eliminated radioactivity associated with the enzyme and the skewing observed in the [ $^{14}\text{C}$ ]glyoxylate elution profile (Figure 6C). Enzyme and nitropropionate were preincubated prior to the addition of [ $^{14}\text{C}$ ]glyoxylate to determine if glyoxylate had to

<sup>2</sup> This value is the product of 0.25 mM, the apparent dissociation constant for the initial E-nitropropionate complex, and  $0.0011/15.8 = 7 \times 10^{-5}$ , the equilibrium constant for reversal of the slow-binding phase of the inhibition. This is equivalent to the ratio of the apparent dissociation rate constant and the second-order rate constant ( $V/K$ ) for association at low nitropropionate ( $1.83 \times 10^{-5} \text{ s}^{-1}/1.05 \times 10^3 \text{ M}^{-1} \text{ s}^{-1}$ ).

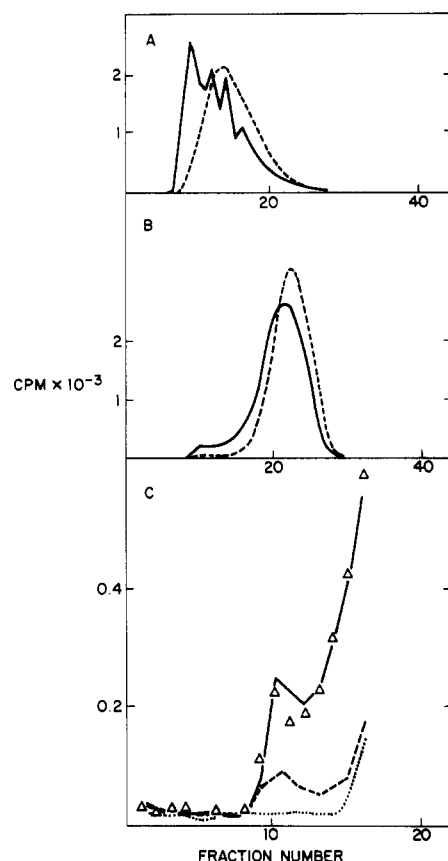


FIGURE 6: Gel filtration profiles for  $[^{14}\text{C}]$ glyoxylate and isocitrate lyase. The samples were applied to a  $0.6 \times 20$  cm column of Sephadex G-25 coarse (A) or G-100 (B and C) and eluted with 0.1 M TAPS-KOH, pH 8, and 10 mM magnesium acetate; 2-drop fractions were collected. (A) Elution of 0.5-mL preincubation mixtures containing 5.3  $\mu\text{M}$  isocitrate lyase, 4  $\mu\text{M}$   $[^{14}\text{C}]$ glyoxylate, 0.1 M TAPS-KOH, pH 8, 10 mM magnesium acetate, 5 mM glutathione, and 1 mM EDTA, with (—) or without (---) 100  $\mu\text{M}$  3-nitropropionate. (B) Elution of 0.5-mL preincubation mixtures as described in (A) with (—) or without (---) 100  $\mu\text{M}$  3-nitropropionate. (C) The leading edge of the profiles shown in (B) and, in addition, one in which glyoxylate was added after preincubation of enzyme and 3-nitropropionate ( $\Delta$ ) and one in which 10 mM unlabeled glyoxylate was added to the preincubation mixture prior to gel filtration (---).

bind to enzyme prior to tight binding of nitropropionate. The elution profile of  $[^{14}\text{C}]$ glyoxylate subsequently obtained was identical with that observed when  $[^{14}\text{C}]$ glyoxylate was added prior to nitropropionate (Figure 6C). Glyoxylate thus binds much tighter to enzyme-nitropropionate than to free enzyme but is in rapid equilibrium compared to the rate of interaction of nitropropionate and isocitrate lyase.

**Substrate Activity of the Nitro Analogue of Isocitrate.** Nitropropionate and glyoxylate or 1-hydroxy-2-nitro-1,3-propanedicarboxylate (the nitropropionate-glyoxylate condensation product and corresponding nitro analogue of isocitrate) were tested as substrates for isocitrate lyase by brief incubation with excess enzyme and then by examination by ion-exchange paper chromatography. Enzyme (0.55 mg, 5.3  $\mu\text{M}$ ) was incubated with  $[^{14}\text{C}]$ glyoxylate (4  $\mu\text{M}$ ) and nitropropionate (200  $\mu\text{M}$ ) or 1-hydroxy-2-nitro-1,3- $[^{14}\text{C}]$ -propanedicarboxylate (4  $\mu\text{M}$ ) in 0.5 mL of 10 mM TAPS-KOH, pH 8, 1 mM magnesium acetate, 1 mM glutathione, and 1 mM EDTA. After 10 min at 25  $^{\circ}\text{C}$ , the enzymic reaction mixtures were quenched with 0.1 g of Dowex 50,  $\text{H}^+$  form. Aliquots of the quenched reaction mixtures were then applied to strips of DE 81 paper ( $1.5 \times 30$  cm), and the chromatographic strips were eluted with 0.1 M Tris-HCl, pH 8, in a descending fashion. After the chromatograms were

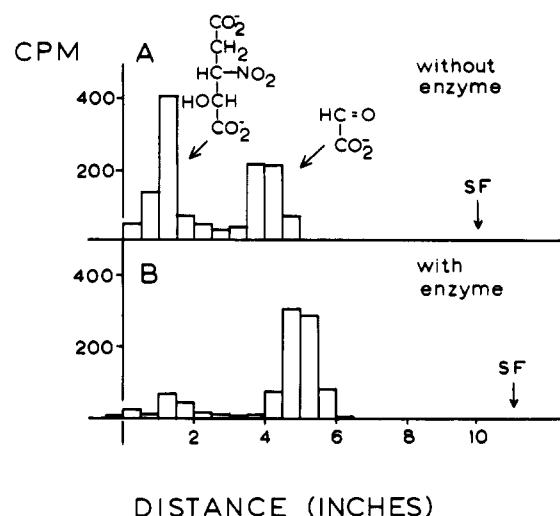


FIGURE 7: Ion-exchange paper chromatograms of 1-hydroxy-2-nitro-1,3- $[^{14}\text{C}]$ propanedicarboxylate after incubation at pH 8 as described in the text without (A) or with (B) isocitrate lyase. The solvent front (SF) is designated with an arrow.

allowed to dry, they were cut into 0.25-in. pieces, each piece was eluted with 1 mL of 1 N HCl, and radioactivity was determined by scintillation counting. 1-Hydroxy-2-nitro-1,3- $[^{14}\text{C}]$ propanedicarboxylate decomposes nonenzymically to nitropropionate and  $[^{14}\text{C}]$ glyoxylate (see Methods), with  $\text{Mg}^{2+}$  greatly stimulating the rate of decomposition (Schloss et al., 1980). Consistent with the instability of the nitro analogue of isocitrate, considerable  $[^{14}\text{C}]$ glyoxylate was formed during incubation in the absence of enzyme (Figure 7a). However, in the presence of enzyme, a far higher proportion of the  $[^{14}\text{C}]$ -labeled nitro analogue was converted into  $[^{14}\text{C}]$ glyoxylate (Figure 7b). 1-Hydroxy-2-nitro-1,3-propanedicarboxylate thus appears to be a substrate for isocitrate lyase. The reverse reaction, enzyme-catalyzed condensation of nitropropionate and  $[^{14}\text{C}]$ glyoxylate, was not observed (data not shown). The nitro analogue of isocitrate was tested as a substrate under multiple turnover conditions in the lactate dehydrogenase coupled assay (Methods). At various isocitrate lyase concentrations (0.1–0.6  $\mu\text{M}$ ), 41% (corrected for slower nonenzymic conversion) of 1-hydroxy-2-nitro-1,3-propanedicarboxylate (0.1 mM) was consistently converted to glyoxylate, when the glyoxylate produced was reduced with NADH by lactate dehydrogenase. Although the nitro analogue contains two asymmetric centers, only the stereoconfiguration of the hydroxyl-bearing carbon is fixed, as the configuration of the carbon bearing the nitro moiety can invert via ionization. Thus, the less than stoichiometric conversion of the racemic nitro analogue to glyoxylate by isocitrate lyase is presumed to be due to only one stereoisomer (with regard to the configuration of the hydroxyl-bearing carbon) having substrate activity.<sup>3</sup> The kinetic constants for the nitro analogue of isocitrate were determined with the lactate dehydrogenase coupled assay. The maximal rate obtained was only 0.8% of that seen for  $D_3$ -isocitrate. Comparable Michaelis constants for the nitro analogue (95  $\mu\text{M}$ ) and  $D_3$ -isocitrate (86  $\mu\text{M}$ ) were obtained.

<sup>3</sup> Since the percent conversion was the same over a 6-fold range of enzyme concentrations, it is unlikely that the less than stoichiometric conversion was caused by enzyme inactivation. Since the enzyme is specific for *threo*- $D_3$ -isocitrate, it seems unlikely that the enzyme would act on the unnatural stereoisomer of the nitro analogue of isocitrate. The somewhat lower conversion than expected (41% vs. 50%) may result from partial decomposition of the stock solution prior to the experiment.

**Inhibition by Other Analogues.** Itaconate and maleate have been reported to bind more tightly to isocitrate lyase than succinate (Rao & McFadden, 1965; Williams et al., 1971; Rittenhouse & McFadden, 1974). Itaconate also exhibits slow-binding inhibition (Rittenhouse & McFadden, 1974). Determination of inhibition constants in the isocitrate dehydrogenase coupled assay (Methods) gave values at pH 8 of 4.2  $\mu\text{M}$ , 0.19 mM, and 0.31 mM for itaconate, maleate, and 3-phosphonopropionate, respectively (data not shown). Michaelis constants of 1.1 mM and 40  $\mu\text{M}$  were obtained for succinate and glyoxylate. The values for itaconate, maleate, succinate, and glyoxylate are comparable to those previously reported (Rittenhouse & McFadden, 1974; Williams et al., 1971; Rao & McFadden, 1965). For determination of the dissociation rate of itaconate, maleate, and 3-phosphonopropionate, enzyme (0.53  $\mu\text{M}$ ) was preincubated (0.1 M TAPS-KOH, pH 8, 10 mM magnesium acetate, 1 mM EDTA, 5 mM glutathione, and 10 mM sodium glyoxylate) with 0.42, 19, or 31 mM inhibitor, respectively, for 30 min prior to 300-fold dilution into an assay mixture. The rate constant obtained for the lag seen in such an assay is equivalent to the dissociation rate constant. Dissociation rates of 0.3 and 4  $\text{min}^{-1}$  were obtained for itaconate and maleate, respectively. Dissociation of 3-phosphonopropionate was too rapid to be determined by this method, and no lag was observed. The dissociation rate obtained for itaconate agrees with the value reported by Rittenhouse & McFadden (1974), 0.2  $\text{min}^{-1}$  at 30 °C and pH 6.8–7.7.

## Discussion

**Slow-Binding Inhibition by 3-Nitropropionate and Other Inhibitors.** Although the interaction of 3-nitropropionate with isocitrate lyase is not amenable to steady-state analysis, several lines of evidence strongly suggest that the time-dependent inhibition is due to slow-binding inhibition that is competitive vs. succinate at the active site (Williams & Morrison, 1979):<sup>4</sup> (1) the inhibition is substantially reversed upon dilution in the presence of high concentrations of succinate, (2) a similar time-dependent inhibition is observed by other reaction-intermediate analogues (itaconate and maleate) which are subject to steady-state analysis (competitive vs. succinate) and are clearly slow-binding inhibitors, (3) isocitrate lyase exhibits higher affinity for glyoxylate after binding nitropropionate and similarly glyoxylate stimulates nitropropionate binding, and (4) there is a striking correlation between tightness of binding and slowness of release of an inhibitor and its structural similarity to the aci form of the succinate carbanion. Thus 3-phosphonopropionate, which binds to isocitrate lyase

about 3-fold tighter than succinate, has a net charge equivalent to that of the succinate carbanion, but its geometry does not mimic the aci form. Maleate is trigonal in the  $\alpha$  position but is also trigonal in the  $\beta$  position and has the same net charge as succinate, rather than its carbanion. Since maleate binds somewhat tighter than 3-phosphonopropionate (about 6-fold tighter than succinate), it appears that geometry is more important than net charge. Itaconate is a still better mimic of the aci form of the succinate carbanion, as it is trigonal in the  $\alpha$  position and tetrahedral in the  $\beta$  position. Itaconate binds substantially tighter than succinate (about 260-fold). The nitronate of 3-nitropropionate best resembles the reaction intermediate. Although its net charge is only equivalent to that of succinate (while both oxygens of the nitro group have negative charge, the nitrogen has a positive charge), the nitronate form of 3-nitropropionate is isosteric with the aci form of the succinate carbanion. Except for phosphonopropionate, the dissociation rates of the inhibitors are slow enough to be readily determined by standard spectrophotometric techniques (4  $\text{min}^{-1}$  for maleate, 0.3  $\text{min}^{-1}$  for itaconate, and  $\leq 0.0011 \text{ min}^{-1}$  for 3-nitropropionate). Thus the closer the structure of an inhibitor is to that of the aci form of succinate carbanion, the tighter it is bound and the slower it is released from the enzyme.

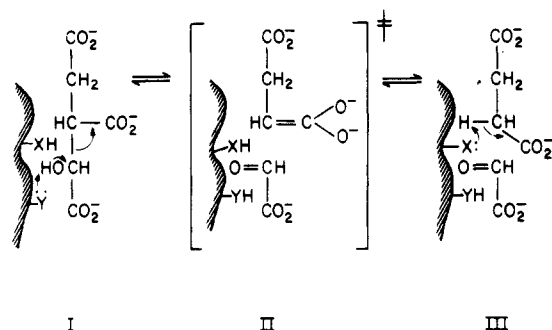
The inhibition constant determined for nitropropionate, 17 nM, is an upper limit. Since the ionized form of nitropropionate is responsible for the time-dependent inhibition, the apparent association rate, 1050  $\text{M}^{-1} \text{ s}^{-1}$ , must be corrected to account for only 9.1% ionization of the carbon acid at pH 8. An association rate constant of  $1.1 \times 10^4 \text{ M}^{-1} \text{ s}^{-1}$ , an overall inhibition constant of 1.5 nM, and a dissociation constant for the initial enzyme-inhibitor complex prior to the slow-binding phase of 23  $\mu\text{M}$  can be calculated for fully ionized nitropropionate. The dissociation rate constant of  $\leq 0.0011 \text{ min}^{-1}$ , which presumably is for release of ionized nitropropionate from the quarternary enzyme- $\text{Mg}^{2+}$ -glyoxylate-nitropropionate complex, is potentially an overestimate due to experimental difficulties in achieving a final, uninhibited steady-state rate. Ionized nitropropionate may thus bind to isocitrate lyase with a dissociation constant less than 1.5 nM.

**Substrate Activity by the Nitro Analogue of Isocitrate and the Acid-Base Chemistry of the Reaction.** In view of the potent and rapid inhibition of isocitrate lyase by 3-nitropropionate, the multiple-turnover substrate activity of the nitro analogue of isocitrate presents a dilemma. Ionized 3-nitropropionate, once tightly bound by isocitrate lyase, is released exceptionally slowly (half-time of release greater than 10 h). However, 3-nitropropionate produced from the nitro analogue of isocitrate during turnover is released relatively rapidly ( $k_{\text{cat}} = 5 \text{ min}^{-1}$ ). We believe the answer to this apparent inconsistency involves the acid-base chemistry of the enzyme. The maximal rate for cleavage ( $V_{\text{max}}$ ) of isocitrate by isocitrate lyase depends on two enzymic groups with apparent pK values of 6.9 and 8.6 (Rogers & McFadden, 1976). The simplest explanation of these data is that cleavage of isocitrate requires two acid-base catalytic groups, one protonated and the other unprotonated. In catalysis of the back-reaction, these two enzymic groups would have the protonation states illustrated as I–III in Scheme II. Thus when isocitrate lyase binds the nitro analogue of isocitrate in a catalytically competent fashion (IV–VI in Scheme II), the enzymic group which normally provides a proton for the succinate carbanion will be protonated. In the subsequent catalytic complex (V) this enzymic group would provide a proton for the nitronate of 3-nitropropionate. However, since the pK of the methylene group

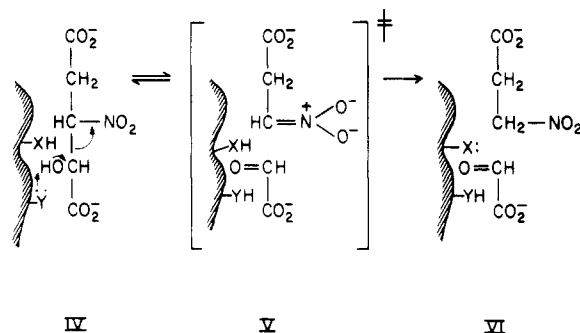
<sup>4</sup> Well-documented examples of slow-binding inhibition include 1-hydroxy-2-nitro-1,3-propanedicarboxylate inhibition of aconitase (Schloss et al., 1980), methotrexate inhibition of dihydrofolate reductase (Williams et al., 1979), nojirimycin, 1-deoxynojirimycin, and acarbose inhibition of sucrose  $\alpha$ -D-glucosylhydrolase (Hanozet et al., 1981), inhibition of hexokinase by various trivalent metal-ATP complexes, notably  $\text{Cr}^{3+}$ - and  $\text{Al}^{3+}$ -ATP (Danenberg & Cleland, 1975; Viola et al., 1980), inhibition of enolase by 2-phosphonoacetohydroxamate and D-tartrate semialdehyde phosphate (Anderson, 1981a,b; Spring & Wold, 1971; Lane & Hurst, 1974), inhibition of adenosine deaminase and adenylyl deaminase by 2'-deoxycoformycin, coformycin, and 1,6-dihydro-6-(hydroxymethyl)purine ribonucleoside (both the nucleosides and their 5'-phosphate esters) (Frieden et al., 1980), itaconate inhibition of isocitrate lyase (Rittenhouse & McFadden, 1974), and inhibition of ribulosebiphosphate carboxylase by 4-carboxy- and 2-carboxy-D-arabinitol 1,5-bisphosphate (Schloss & Lorimer, 1982; Pierce et al., 1980). In most of these examples the inhibitor is structurally similar to a presumed reaction intermediate. Slow-binding inhibition appears frequently to be associated with analogues of reaction intermediates or transition states.

Scheme II

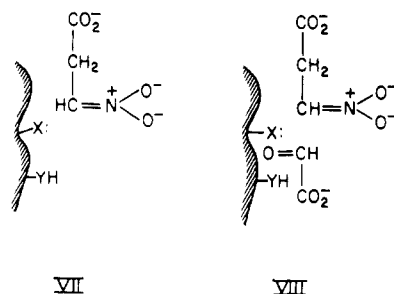
## SUBSTRATE:



## NITRO SUBSTRATE:



## NITRO INHIBITOR:



of 3-nitropropionate is substantially lower than that of succinate (9 compared with about 25), protonation of the nitronate would proceed far less readily, explaining why the nitro analogue is such a sluggish substrate.

3-Nitropropionate, by contrast, appears to bind to the enzyme in a fashion analogous to succinate, with the group responsible for abstracting a proton from succinate in its basic form (complexes VII and VIII, Scheme II). However, 3-nitropropionate has the same overall charge as succinate only in its nitronate form, and since a net 2- charge is apparently essential for binding (Rao & McFadden, 1965), we see binding only of the nitronate, and not of the protonated form which is isosteric with succinate. The enzyme has evolved to stabilize the aci form of succinate carbanion with respect to succinate by at least 15 orders of magnitude (the degree to which the pK of the CH<sub>2</sub> group has been decreased), and when presented with a molecule already isosteric with the acicarboxylate, the available energy leads to extremely tight binding, the necessary consequence of which is a slow rate constant for inhibitor release. Since the group responsible for protonating the succinate carbanion is in its basic form in complexes VII and VIII, nitropropionate cannot be protonated to facilitate its release, as occurs when it is formed from the nitro analogue of isocitrate, except at the very slow rate at which a proton can penetrate the closed catalytic form of the enzyme which

the complex with the inhibitor apparently assumes.<sup>5</sup>

**Kinetic Mechanism of Isocitrate Lyase.** Since itaconate is competitive vs. succinate, but uncompetitive vs. either glyoxylate or isocitrate, and glyoxylate is competitive and succinate noncompetitive vs. isocitrate (Williams et al., 1971), it would appear that isocitrate lyase has an ordered kinetic mechanism, with glyoxylate necessary for succinate binding. However, as evidenced by its competition with nitropropionate, succinate can bind to free enzyme with an apparent dissociation constant only about 2-fold greater than its *K<sub>m</sub>*. Binding of [<sup>14</sup>C]succinate and [<sup>14</sup>C]itaconate to free enzyme has been reported (Rittenhouse & McFadden, 1974). Nitropropionate binds more rapidly to enzyme-glyoxylate than to free enzyme. Whether there is a corresponding decrease in the rate of nitropropionate release from enzyme-glyoxylate relative to free enzyme is not known. Glyoxylate binds more tightly to enzyme-nitropropionate than to free enzyme. Collectively, these observations suggest that isocitrate lyase has a random kinetic mechanism with a preferred pathway (glyoxylate binding first). Williams et al. (1971) reached a similar conclusion.

## References

- Anderson, V. E. (1981a) Ph.D. Dissertation, University of Wisconsin.  
 Anderson, V. E. (1981b) *Fed. Proc., Fed. Am. Soc. Exp. Biol.* 40, 1657.  
 Beach, R. L., Aogaichi, T., & Plaut, G. W. E. (1977) *J. Biol. Chem.* 252, 2702-2709.  
 Beevers, H. (1979) *Annu. Rev. Plant Physiol.* 30, 159-193.  
 Bellion, E., & Kelley, R. L. (1979) *J. Bacteriol.* 138, 519-522.  
 Cleland, W. W. (1967) *Adv. Enzymol. Relat. Areas Mol. Biol.* 29, 1-32.  
 Danenberg, K. D., & Cleland, W. W. (1975) *Biochemistry* 14, 28-39.  
 DeMaggio, A. E., Greene, C., Unal, S., & Stetler, D. A. (1979) *Science (Washington, D.C.)* 206, 580-582.  
 Frieden, C., Kurz, L. C., & Gilbert, H. R. (1980) *Biochemistry* 19, 5303-5309.  
 Gawron, O., & Mahajan, K. P. (1966) *Biochemistry* 5, 2335-2342.  
 Gemmrich, A. R. (1979) *Phytochemistry* 18, 1143-1146.  
 Gemmrich, A. R. (1980) *Z. Pflanzenphysiol.* 97, 153-160.  
 Hanozet, G., Pircher, H., Vanni, P., Oesch, B., & Semenza, G. (1981) *J. Biol. Chem.* 256, 3703-3711.  
 Henson, C. P., & Cleland, W. W. (1967) *J. Biol. Chem.* 242, 3833-3838.  
 Khan, F. R., & McFadden, B. A. (1979) *Plant Physiol.* 64, 228-231.  
 Khan, F. R., & McFadden, B. A. (1980) *FEBS Lett.* 115, 312-314.  
 Kornberg, H. L., & Elsdén, S. R. (1961) *Adv. Enzymol. Relat. Areas Mol. Biol.* 23, 401-470.  
 Krupka, R. M., & Towers, G. H. N. (1958) *Nature (London)* 181, 335-336.  
 Lane, R. H., & Hurst, J. K. (1974) *Biochemistry* 13, 3292-3297.  
 McFadden, B. A. (1969) *Methods Enzymol.* 13, 163-170.

<sup>5</sup> In complex VIII glyoxylate is apparently much more free to dissociate than nitropropionate. Further, its release is much faster than proton penetration to the key catalytic base which protonates aci forms. This difference is presumably the result of the different amounts of energy the enzyme normally brings to bear on the two substrates to convert them into catalytically active forms. Far more energy must be focused on succinate to convert it to a carbanion than on glyoxylate. It is presumably the geometry of the active site that prevents proton access to the catalytic group involved in succinate ionization.



- McFadden, B. A., & Purohit, S. (1977) *J. Bacteriol.* 131, 136-144.
- McFadden, B. A., Rose, I. A., & Williams, J. O. (1972) *Arch. Biochem. Biophys.* 148, 84-88.
- McKinley, M. P., & Trelease, R. N. (1978) *Protoplasma* 94, 249-261.
- Ormerod, J. G., Ormerod, K. S., & Gest, H. (1961) *Arch. Biochem. Biophys.* 94, 449-463.
- Patel, T. R., & McFadden, B. A. (1978a) *Nematologica* 24, 51-62.
- Patel, T. R., & McFadden, B. A. (1978b) *Exp. Parasitol.* 44, 262-268.
- Pierce, J., Tolbert, N. E., & Barker, R. (1980) *Biochemistry* 19, 934-942.
- Porter, D. J. T., & Bright, H. J. (1980) *J. Biol. Chem.* 255, 4772-4780.
- Rao, G. R., & McFadden, B. A. (1965) *Arch. Biochem. Biophys.* 112, 294-303.
- Rittenhouse, J. O. W., & McFadden, B. A. (1974) *Arch. Biochem. Biophys.* 163, 79-86.
- Rogers, J. E., & McFadden, B. A. (1976) *Arch. Biochem. Biophys.* 174, 695-704.
- Schloss, J. V. (1981) *Fed. Proc., Fed. Am. Soc. Exp. Biol.* 40, 1657.
- Schloss, J. V., & Lorimer, G. H. (1982) *J. Biol. Chem.* 257, 4691-4694.
- Schloss, J. V., Porter, D. J. T., Bright, H. J., & Cleland, W. W. (1980) *Biochemistry* 19, 2358-2362.
- Spring, T. G., & Wold, F. (1971) *Biochemistry* 10, 4655-4660.
- Viola, R. E., Morrison, J. F., & Cleland, W. W. (1980) *Biochemistry* 19, 3131-3137.
- Williams, J. O., Roche, T. E., & McFadden, B. A. (1971) *Biochemistry* 10, 1384-1390.
- Williams, J. W., & Morrison, J. F. (1979) *Methods Enzymol.* 63A, 437-467.
- Williams, J. W., Morrison, J. F., & Duggleby, R. G. (1979) *Biochemistry* 18, 2567-2573.

## Purification and Partial Characterization of Rat Liver Folate Binding Protein: Cytosol I<sup>†</sup>

Robert J. Cook and Conrad Wagner\*

**ABSTRACT:** The high molecular weight folate binding protein of rat liver cytosol has been purified to apparent homogeneity. Purification was achieved by using a combination of gel filtration, *O*-(diethylaminoethyl)cellulose chromatography, and affinity chromatography. This folate binding protein was initially identified during purification by an *in vivo* labeling procedure involving intraperitoneal injection of [<sup>3</sup>H]folic acid prior to sacrifice and subsequently by its ability to bind naturally reduced [<sup>3</sup>H]folate polyglutamates *in vitro*. A molecular

weight of 210 000 was estimated by gel chromatography. This is distinct from the trifunctional formyl-methenyl-methylene synthetase of rat liver which has a molecular weight of 225 000. Sodium dodecyl sulfate electrophoresis revealed a single band with a molecular weight of about 100 000 which suggests the native protein is composed of two identical subunits. The partially purified protein contains bound tetrahydropteroyl-pentaglutamate.

Zamierowski & Wagner (1974, 1977) reported the presence of three proteins in rat liver cytosol which contained tightly bound endogenous folates. These proteins were separated by chromatography using Sephadex G-150 and were referred to as folate binding proteins of cytosol I, II, and III (FBP-CI, FBP-CII, and FBP-CIII, respectively).<sup>1</sup> FBP-CII has previously been purified and characterized (Suzuki & Wagner, 1980), and its distribution in various rat tissues has been measured by radioimmunoassay (Cook & Wagner, 1981). FBP-CIII was shown to be associated with dihydrofolate reductase (5,6,7,8-tetrahydrofolate:NADP<sup>+</sup> oxidoreductase, EC 1.5.1.3) activity and has an estimated molecular weight of 25 000. A fourth folate binding protein was identified in sonicated extracts of rat liver mitochondria and was referred to as mitochondrial folate binding protein (MFBP; Zamierowski & Wagner, 1974, 1977). MFBP was recently resolved

into two related enzymes which have been purified and characterized. They are dimethylglycine dehydrogenase (EC 1.5.99.2) and sarcosine dehydrogenase (EC 1.5.99.1) (Wittwer & Wagner, 1980, 1981a,b).

We now report the purification to apparent homogeneity and partial characterization of FBP-CI. This protein was previously shown to have an approximate molecular weight of about 350 000 by gel filtration of liver cytosol. Purification

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<sup>1</sup> Abbreviations: FBP-CI, folate binding protein cytosol I; FBP-CII, folate binding protein cytosol II; FBP-CIII, folate binding protein cytosol III; MFBP, mitochondrial folate binding protein; PteGlu, pteroylglutamic acid (folic acid); H<sub>2</sub>PteGlu, 7,8-dihydropteroylglutamic acid (dihydrofolic acid); H<sub>4</sub>PteGlu, 5,6,7,8-tetrahydropteroylglutamic acid (tetrahydrofolic acid); CH<sub>3</sub>, methyl; HCO, formyl; CH<sub>2</sub>, methylene; PteGlu<sub>2</sub>, pteroyldiglutamic acid; PteGlu<sub>3</sub>, pteroyltriglutamic acid; PteGlu<sub>5</sub>, pteroylpentaglutamic acid; PteGlu<sub>6</sub>, pteroylhexaglutamic acid; *p*-ABG, (*p*-aminobenzoyl)glutamic acid; FMMS or formyl-methenyl-methylene synthetase (combined), formyltetrahydrofolate synthetase (EC 6.3.4.3), methenyltetrahydrofolate cyclohydrolase (EC 3.5.4.9), and methylene-tetrahydrofolate dehydrogenase (EC 1.5.1.5); BSA, bovine serum albumin; EDAC, 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide hydrochloride; NaDodSO<sub>4</sub>, sodium dodecyl sulfate; DEAE-cellulose, *O*-(diethylaminoethyl)cellulose; TEAE-cellulose, *O*-(triethylaminoethyl)cellulose; TBAP, tetrabutylammonium phosphate; Tris, tris(hydroxymethyl)aminomethane.