# Turnover and Inactivation of Bacterial Citrate Lyase with 2-Fluorocitrate and 2-Hydroxycitrate Stereoisomers†

Steven E. Rokita and Christopher T. Walsh*

ABSTRACT: Bacterial citrate lyase catalytically cleaves the carbon skeleton of the naturally occurring fluorocitrate isomer (−)-*erythro*-2-fluorocitrate (2*R*,3*R*) with the same regiospecificity as with citrate cleavage. The carbon-skeleton cleavage rate of this analogue is 1% of the $V_{max}$ for citrate turnover, and it binds to the enzyme with a $K_I$ value of 4.9 μM, 300-fold lower than the $K_m$ of citrate. Cleavage of the analogue yields oxalacetate and a fluoroacetyl form of the resting citrate lyase (vs. the acetyl form after citrate turnover). (−)-*erythro*-2-Fluorocitrate inactivates citrate lyase ca. 500-fold more frequently than citrate (ca. 20 vs. 6500–12000 catalytic cycles per enzyme inactivation), producing the deacetylated, inactive form of the enzyme. The (+)-*erythro*-2-fluorocitrate (2*S*,3*S*) is also cleaved very slowly (0.05% the $V_{max}$ for citrate) but does not cause measurable enzyme inactivation (over 100 min). Even when the turnover rate of citrate lyase is reduced 2000-fold as above, the regiospecificity of processing remains the same as that of citrate, yielding β-fluorooxalacetate and the acetyl form of resting citrate lyase. Among the four diastereomers of 2-hydroxycitrate, three forms—(+)-threo, (−)-threo, and (−)-erythro—are catalytically cleaved by citrate lyase. The regiospecificity of processing in all cases is invariant and identical with that of citrate cleavage. (−)-*erythro*-2-Hydroxycitrate (2*R*,3*S*) and (−)-*threo*-2-hydroxycitrate (2*S*,3*S*) yield oxalacetate and the glycolyl form of resting citrate lyase in the presence of either zinc or magnesium ions. (+)-*threo*-2-Hydroxycitrate (2*R*,3*R*) yields β-hydroxyoxalacetate and the acetyl form of citrate lyase if magnesium is present, but no catalytic activity is evident in the presence of zinc. The (+)-threo and (−)-erythro isomers inactivate citrate lyase with partition ratios of less than 100 catalytic cycles per enzyme inactivation. (+)-*erythro*-2-Hydroxycitrate (2*S*,3*R*) appears to be a completely efficient turnover-dependent inactivator with a $k_{inact}$ of 0.68–1.1 $min^{-1}$ depending on the metal ion present. This isomer effects net hydrolysis of the active, acetyl form of the enzyme to a catalytically inactive form without producing any detectable carbon-skeleton cleavage. Enzyme inactivation induced by all of the above citrate analogues may follow a common path. The hydrolysis of a catalytic intermediate, the mixed-acid anhydride, could be the sole cause of enzyme inactivation.

Fluorine has become a common substituent in pharmacologically active organic compounds [for reviews, see Peters (1972), Filler (1976), and Walsh (1983)]. A broad understanding of the enzymic reactions at or adjacent to carbon–fluorine bonds is necessary for predicting the metabolism and toxicity of fluorinated substrate analogues. This paper reports the interactions between bacterial citrate lyase (EC 4.1.1.3.6) and the (+)- and (−)-*erythro*-2-fluorocitrate enantiomers and focuses on the stereochemistry of carbon-skeleton cleavage and turnover-dependent inactivation. Citrate was chosen as the parent compound for two major reasons. First, predictions can be made and tested concerning the metabolism of citrate derivatives because the enzymes that process citrate have been well characterized (Srere, 1975; Glusker, 1971). Second, the ability of a fluorine substituent to mimic the properties of a substrate hydrogen or hydroxyl group can be studied within a set of citrate derivatives [see also Marletta et al. (1981) and Rokita et al. (1982)].

Replacement of a methylene hydrogen of citrate with fluorine will not greatly affect the steric properties of this compound (C–H bond length is 1.09 Å in methane; C–F bond length is 1.39 Å in fluoromethane) but will increase the polarity of the substituted carbon [see p 95 in Peters (1972)]. The polarity of fluorine and its ability to accept hydrogen bonds more closely resemble the properties of a hydroxyl group than those of the parent hydrogen in citrate. To compare the reactivity of a fluorine vs. a hydroxyl-group substitution at C-2 of citrate, this paper also details the interactions between the 2-hydroxycitrate stereoisomers and citrate lyase. Previously, the reactivities of these hydroxylated analogues with citrate lyase were briefly surveyed by Sullivan et al. (1977a).

When a substituent replaces one of the methylene hydrogens in the prochiral citrate parent, two chiral centers are formed as shown in Scheme I—one at the substituted carbon and the other at carbon 3.[1] The stereochemistry of these analogues defines their biological activity. Only the (−)-erythro stereoisomer of 2-fluorocitrate is toxic (Fanshier et al., 1964). This stereoisomer is produced in vivo from fluoroacetate by what Peters (1972) described as the "lethal synthesis". Two hydroxylated citrate derivatives occur in nature; (+)-*erythro*-2-hydroxycitrate can be isolated from the hibiscus plant, and (−)-*threo*-2-hydroxycitrate can be isolated from garcinia fruit (Lewis & Neelakantan, 1965). Only (−)-*threo*-2-hydroxycitrate inhibits the formation of triglycerides and cholesterol in vivo (Sullivan et al., 1977b).

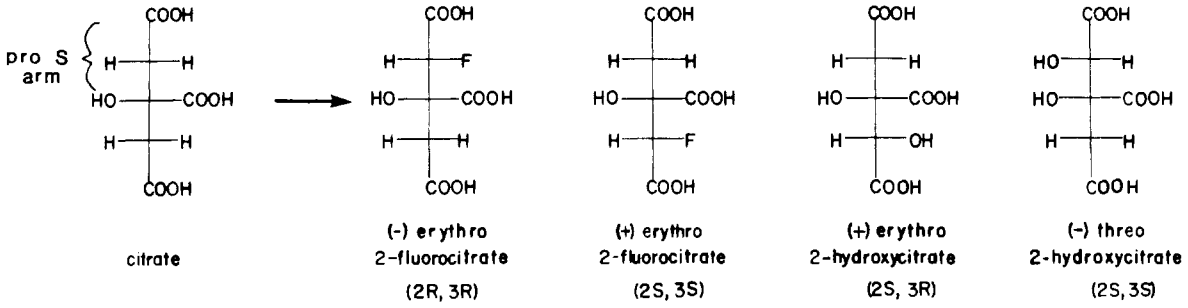
The activity of each 2-fluoro- and 2-hydroxycitrate stereoisomer with citrate lyase addressed here is consistent with the proposed mechanism of catalysis. This enzyme is unusual

† From the Departments of Chemistry and Biology, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139. *Received December 17, 1982; revised manuscript received March 3, 1983.* This investigation was supported in part by National Institutes of Health Grant GM 20011.

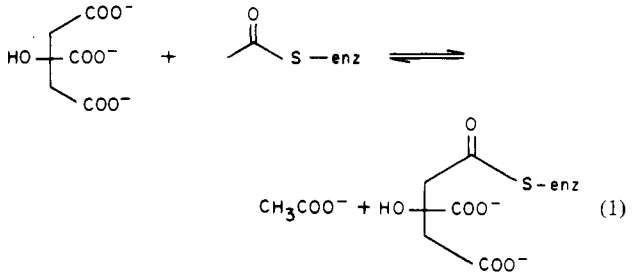
[1] The stereochemistry of the citrate derivatives is designated by the erythro and threo nomenclature to illustrate possible steric equivalence between 2-fluoro- or 2-hydroxycitrate stereoisomers. The (−)-*erythro*-2-fluorocitrate, for example, is sterically equivalent to the (−)-*erythro*-2-hydroxycitrate. Furthermore, the numbering system for the citrate carbon skeleton used here is a historic and widely quoted one. The Cahn–Ingold–Prelog stereochemical designations are added parenthetically. Stereoequivalent fluorinated and hydroxylated citrate derivatives will not necessarily have the same *R*/*S* assignment due to the priority system for carbon-bound substituents. An alternative nomenclature for derivatives of citrate (not shown) has been developed from the parent citrate molecule (Glusker & Srere, 1973).

0006-2960/83/0422-2821$01.50/0 

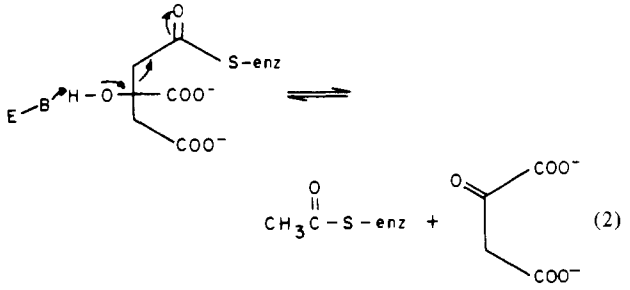
Scheme I




because it contains an essential acetyl group for activity (Buckel et al., 1971; Srere et al., 1972). This moiety is bound as a thiol ester to a modified coenzyme A covalently attached to one of the three subunits of citrate lyase. Another subunit of this enzyme catalyzes acyl exchange between the active site bound acetyl group and citrate (eq 1). The third subunit, in

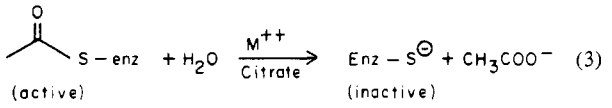



the presence of certain divalent metals, then catalyzes the carbon-skeleton cleavage of citryl-S-lyase[2] (eq 2), releasing




free oxalacetate and regenerating the acetyl enzyme (Dimroth & Eggerer, 1975a).

Citrate lyase also autoinactivates (eq 3) in the presence of




citrate. This process is attributed to a metal-dependent hydrolysis during the transacylation step resulting in a net deacetylation of the enzyme (Singh & Srere, 1971). All kinetic data reported here have been measured in the presence of both $Mg^{2+}$ and $Zn^{2+}$ in order to facililate a comparison of our data and those previously reported in the presence of only $Mg^{2+}$ or $Zn^{2+}$.

[2] Abbreviations: MDH, malate dehydrogenase; unit, enzyme unit defined to be the quantity of enzyme necessary to transform 1 μmol of substrate in 1 min; $NAD^+$ and NADH, nicotinamide adenine dinucleotide, oxidized and reduced, respectively; cpm, counts per minute; HPLC, high-performance liquid chromatography; acetyl-S-lyase, the active form of citrate lyase with an acetylated, covalently bound modified coenzyme A; HS-lyase, citrate lyase (inactive) with the acetyl group removed; citryl-S-lyase, 2-hydroxycitryl-S-lyase, 3-fluoro-3-deoxycitryl-S-lyase, 2-fluorocitryl-S-lyase, fluoroacetyl-S-lyase, and glycolyl-S-lyase, citrate lyase with the appropriate acyl moiety attached through a thiol ester to the coenzyme A like group.

## Materials and Methods

The synthesis of β-fluorooxalacetate has been described by Goldstein et al. (1978). The (+)- and (−)-*erythro*-2-fluorocitrate enantiomers were synthesized and resolved according to known procedures (Dummel & Kun, 1969). [**Caution**: handle (−)-*erythro*-2-fluorocitrate with extreme care. It is highly toxic.] The resolved (−)- and (+)-*threo*-2-hydroxycitrate and (−)- and (+)-*erythro*-2-hydroxycitrate stereoisomers were the gift of Dr. Ann Sullivan from the Department of Biochemical Nutrition of Hoffmann-La Roche Inc. (Nutley, NJ). (4*R*)-[4-$^3$H]NADH (1.7 μCi/μmol) was the gift of Dr. C. Ryerson from this laboratory and was prepared according to the procedures of Oppenheimer et al. (1971). All other chemicals and reagents were of the highest quality commercially available.

Citrate lyase from *Klebsiella aerogenes* was purchased from Sigma Chemical Co. in a preparation containing, by weight, 24% bovine serum albumin, 48% saccharose, 24% citrate lyase (6.1 units/mg), and 4% $MgSO_4 \cdot 7H_2O$. This mixture was used without purification of citrate lyase because this enzyme is less stable when the other components are removed. This enzyme was assayed by coupling the formation of oxalacetate (or its fluoro or hydroxy derivatives) to its subsequent reduction with NADH and MDH (Bergmeyer, 1974). All kinetic assays were performed at 25 °C and buffered with 100 mM triethanolamine at pH 7.6. Between 48 and 240 μg of enzyme protein (from the citrate lyase preparation) was used for $k_{cat}$ determinations; the concentrations of the citrate derivatives were at least 10-fold greater than their $K_I$ [this paper and Sullivan et al. (1977a)] to approximate maximum rate conditions. Turnover inactivation was never too fast to obscure the initial rate of carbon-skeleton cleavage over the first 2 min. The concentration of metal ions present in the assays (2 mM) exceeded, in every case, the concentrations of the carbon substrate. Because $Mg^{2+}$ was present in the Sigma preparation of citrate lyase, assays containing zinc were contaminated with magnesium.

A $K_I$ value for (+)-*erythro*-2-fluorocitrate was calculated from its competitive inhibition of citrate cleavage (determined by a Lineweaver–Burk plot). The $K_I$ and $k_{inact}$ values for the time-dependent inactivation of citrate lyase by (−)-*erythro*-2-fluorocitrate were determined as described for 3-fluoro-3-deoxycitrate (Rokita et al., 1982). The specific activity of citrate lyase (96 μg of enzyme protein) incubations containing varying concentrations of inhibitor at 25 °C in 200 μL of 1 and 2 mM $Zn^{2+}$–100 mM triethanolamine, pH 7.6, was monitored over time. A $K_I$ and $k_{inact}$ were based on a double-reciprocal plot of the observed $k_{inact}$ vs. the inhibitor concentration. Values of $k_{inact}$ for (+)-*threo*- and (+)-*erythro*-2-hydroxycitrate were determined from the decrease in the specific activity of citrate lyase (240 μg) when incubated in a 200-μL solution of 2 mM $Zn^{2+}$–80 mM triethanolamine, pH 7.6, and inhibitor concentrations 10 times their published

$K_I$ (Sullivan et al., 1977a). The $k_{inact}$ value for (+)-*erythro*-2-hydroxycitrate in the presence of 2 mM $Mg^{2+}$ was too large to measure as above; instead, a $k_{inact}$ was extrapolated as in the case of the (−)-*erythro*-fluoro derivative. The turnover inactivation constants, $k_{inact}$, and partition ratio (catalytic turnovers per enzyme inactivation), were calculated for citrate and its analogues from the initial rate of carbon-skeleton cleavage and the total number of moles of substrate cleaved per mole of catalytically competent active site (see legend of Table II). Although native citrate lyase ($M_r$ of 550 000) contains six subunit complexes (Bowen & Mortimer, 1971), the above calculations assume that only four of these complexes are functional (Dimroth & Eggerer, 1975b; Singh et al., 1975). The addition of 2–3 μL of reagent-grade acetic anhydride to a routine activity assay was sufficient for the acetic anhydride reactivation of citrate lyase (Buckel et al., 1971; Srere et al., 1972; Rokita et al., 1982).

The α-keto acid cleavage products of citrate and its derivatives were reduced in situ by (4*R*)-[4-$^3$H]NADH and MDH. A 600-μL enzyme incubation of 0.3–0.8 mM $ZnCl_2$, 70 μM [$^3$H]NADH, 3 mM citrate analogue, 80 mM triethanolamine, pH 7.6, 1.2 mg of citrate lyase, and 100 units of MDH was quenched after 2–6 h by the addition of HCl to a final pH of 3–4. Before the mixture was separated by anion-exchange HPLC (see Figure 1), the nucleotides were removed from this incubation with activated charcoal, followed by filtration through a 0.45-μm Millex-HA aqueous filter (Millipore Corporation), and 1 μmol of malate and 3 μmol of tartrate were added as nonradioactive standards.

## Results and Discussion

*Citrate Lyase Catalyzed Cleavage of 2-Fluoro- and 2-Hydroxycitrate Stereoisomers: Product Identification.* Before the chemistry of the 2-fluoro- and 2-hydroxycitrate stereoisomers can be discussed, their catalytically competent alignment in the active site of citrate lyase must be defined. Carrell et al. (1970), Sullivan et al. (1977a), and Stallings et al. (1979) have suggested that a fluoro- or hydroxyl-group substitution at the methylene position of citrate may cause these derivatives to bind citrate-processing enzymes in a manner not analogous to the binding of citrate. However, the regiospecificity of citrate synthase (EC 4.1.3.7) and the mammalian ATP citrate lyase (EC 4.1.3.8) catalysis is constant for citrate [for a review, see Srere (1975)] and the 2-fluorocitrate stereoisomers (Fanshier et al., 1964; Marletta et al., 1981). These enzymes catalyze a reversible aldol-type condensation in which the carbon bond cleaved or produced always occurs at the arm corresponding to the *pro-S* arm of citrate.

Rokita et al. (1982) reported a MDH-coupled citrate lyase activity for both of the *erythro*-2-fluorocitrates, and Sullivan et al. (1977a) reported that three of the four 2-hydroxycitrate stereoisomers were also substrates using the same MDH-coupled assay. However, MDH will catalyze the reduction of all of the possible α-keto acid products, oxalacetate, β-fluorooxalacetate (Marletta et al., 1981), and β-hydroxyoxalacetate (Sullivan et al., 1977a). The stereochemistry of citrate lyase turnover and, therefore, the orientation of productive binding can only be determined in each case after the cleavage products are identified.

β-Fluorooxalacetate, produced by citrate lyase, is sufficiently stable to be detected by HPLC without prior derivatization. The other possible four-carbon keto acid fragments were successfully identified by HPLC only after in situ reduction with (4*R*)-[4-$^3$H]NADH and MDH (see Figure 1).

Figure 2 summarizes the cleavage products identified after

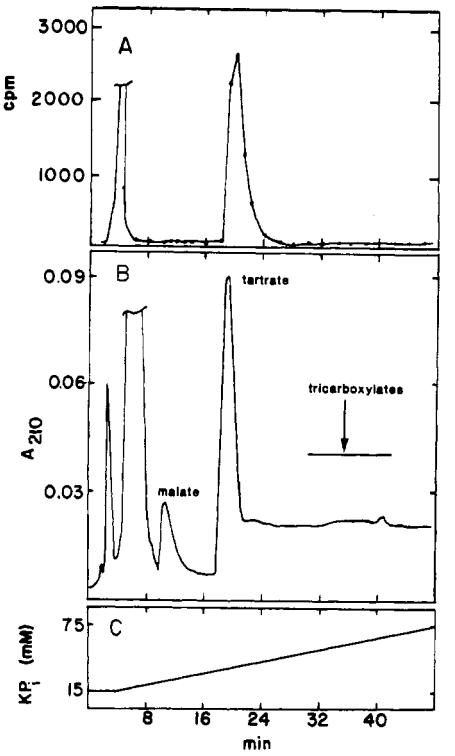



FIGURE 1: HPLC separation of citrate lyase processed (+)-*threo*-2-hydroxycitrate after reduction with [$^3$H]NADH. The citrate lyase incubation (described under Materials and Methods) was analyzed on a Waters Associates HPLC (including a 660 gradient programmer) equipped with a Micromeritics 786 variable-wavelength detector and an analytic Whatman Partisil SAX strong anion exchange column (protected by a $C_{18}$ guard column). (A) HPLC fractions (2 mL) were mixed with 15 mL of scintillant and analyzed for tritium content on a Beckman LS-100 scintillation counter. Note: there is ca. a 3-mL dead volume between the variable-wavelength detector flow cell and the fraction collector. (B) Nonradioactive malate and tartrate carrier were added to the enzyme incubations and detected by their absorbance at 210 nm. (C) The HPLC base line rose in parallel with the indicated gradient of potassium phosphate, pH 3.8, with a flow rate of 2 mL/min.

citrate lyase turnover of the 2-fluoro- and 2-hydroxycitrates. Similar to citrate synthase and ATP citrate lyase (Fanshier et al., 1964; Marletta et al., 1981), citrate lyase processes the fluorinated substrates with the same regiospecificity as citrate. If cleavage with the reverse regiospecificity competes during catalysis, it must occur less than 2% of the time. The cleavage of (−)-*erythro*-2-fluorocitrate (2*R*,3*R*) yields oxalacetate, suggesting that the enzyme must be left at the end of the first catalytic cycle in the fluoroacetyl form (fluoroacetyl-S-lyase) instead of the acetyl form (acetyl-S-lyase). Citrate lyase remains active in this form because the modified enzyme initiates further catalytic cycles.

In the 2-hydroxycitrate series (Figures 1 and 2), the only productive binding for these analogues is one that mimics that of citrate [confirming the predictions of Sullivan et al. (1977a)]. For the three stereoisomers that are substrates for citrate lyase, cleavage always occurs at what corresponds to the *pro-S* arm of citrate. Just as the catalytic turnover of (−)-*erythro*-2-fluorocitrate demonstrates that fluoroacetate can substitute for the active site acetyl group, the catalytic turnover of (2*R*,3*S*)-(−)-*erythro*- and (2*R*,3*R*)-(−)-*threo*-2-hydroxycitrate demonstrates that a glycolyl form of citrate lyase (glycolyl-S-lyase) is also catalytically active.

Only one alignment of the six-carbon substrate will undergo catalytic cleavage despite the variety of ways citrate and its derivatives may chelate divalent metal ions (Stallings et al., 1979, 1980; Glusker, 1980). Neither a fluoro nor a hydroxy

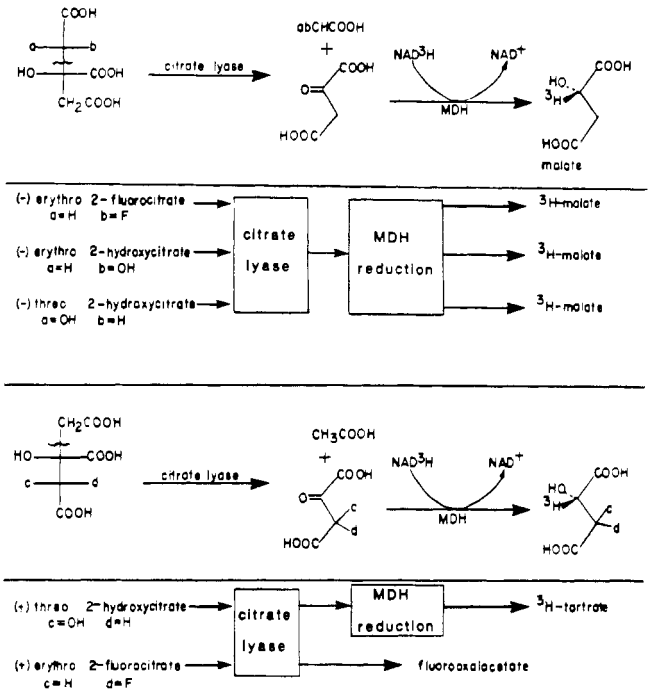



FIGURE 2: Citrate lyase cleavage pattern with citrate analogues. The regiospecificity of citrate lyase processing is summarized here by identifying the products of catalytic turnover either directly or after reduction with (4*R*)-[4-$^3$H]NADH and MDH. Enzyme incubations are described under Materials and Methods and were analyzed by HPLC as illustrated in Figure 1. The metabolite that contained the tritium is listed on the right. $\beta$-Fluorooxalacetate was identified directly by coelution with a standard solution of $\beta$-fluorooxalacetate (using the same HPLC columns but an isocratic elutant of 100 mM potassium phosphate, pH 3.8).

substituent realigns the substrate–enzyme binding geometry to allow for any loss of stereospecific processing, even though the rate of catalysis may be decreased more than a 1000-fold by the added substituent (see below).

*Binding Recognition and Catalytic Carbon-Bond Cleavage of the 2-Fluorocitrates with Citrate Lyase.* The chemical effects of the fluorine substituent in 2-fluorocitrate during substrate binding and turnover can now be divided into the effects of a fluorine geminal, (−)-*erythro*-2-fluorocitrate (2*R*,3*R*), and vicinal, (+)-*erythro*-2-fluorocitrate (2*S*,3*S*), to the carbon bond cleaved (Figure 2). Because these analogues are processed very slowly (see below), active site binding was initially assessed by measuring competitive inhibition of citrate turnover. The $K_I$ for (+)-*erythro*-2-fluorocitrate is 86 $\mu$M, almost 20-fold lower than the $K_m$ found experimentally for citrate ($K_m$ = 1.6 mM). A $K_I$ from competitive inhibition assays could not be determined for (−)-*erythro*-2-fluorocitrate because it quickly and irreversibly inactivated citrate lyase. Instead, a $K_I$ of 4.9 $\mu$M for the (−)-erythro isomer was derived from time-dependent inactivation studies. This is almost 3 orders of magnitude lower than the citrate $K_m$.

The $k_{cat}$ values for 2-fluorocitrate turnover, listed in Table I, are only 0.05% [(+)-erythro isomer] to 1% [(−)-erythro isomer] the $k_{cat}$ for citrate. (−)-*erythro*-2-Fluorocitrate (with the fluorine geminal to the bond broken) is cleaved 20-fold faster than (+)-*erythro*-2-fluorocitrate (with the vicinal fluorine). However, mammalian ATP citrate lyase exhibits the exact opposite order of reactivity with these stereoisomers (Marletta et al., 1981). Clearly, the fluorine substituent at either position does not have the same effect during turnover of these enzymes. Those characteristics of a fluorine substitution (e.g., inductive effect) that remain constant during the turnover of both enzymes cannot then explain the relative activity of the 2-fluorocitrates.

Table I: Carbon-Skeleton Cleavage Rates of Citrate Derivatives Catalyzed by Citrate Lyase[a]

| compd | turnover ($k_{cat}$) with 2 mM $Zn^{2+}$ ($min^{-1}$) | turnover ($k_{cat}$) with 2 mM $Mg^{2+}$ ($min^{-1}$) |
|---|---|---|
| citrate | 750 | 1100 |
| (−)-*erythro*-2-fluorocitrate | 6.3 | 2.6 |
| (+)-*erythro*-2-fluorocitrate | 0.38[b] | 0.63 |
| (−)-*erythro*-2-hydroxycitrate | 1.2 | 1.0 |
| (+)-*erythro*-2-hydroxycitrate | $<10^{-4}$ | <0.1 |
| (−)-*threo*-2-hydroxycitrate | 0.35 | 0.45 |
| (+)-*threo*-2-hydroxycitrate | <0.1 | 2.3 |

[a] Malate dehydrogenase (MDH) coupled activity assays were used (see Materials and Methods). The upper limits for the (+)-*threo*-2-hydroxycitrate ($Zn^{2+}$) and (+)-*erythro*-2-hydroxycitrate ($Mg^{2+}$) turnover were dictated by the sensitivity of the MDH assay. The limit for the (+)-*erythro*-2-hydroxycitrate ($Zn^{2+}$) rate was determined by the sensitivity of the HPLC assay. [b] These low turnover numbers do not reflect trace impurities in each stereoisomer as shown by Marletta et al. (1981) and Sullivan et al. (1977a).

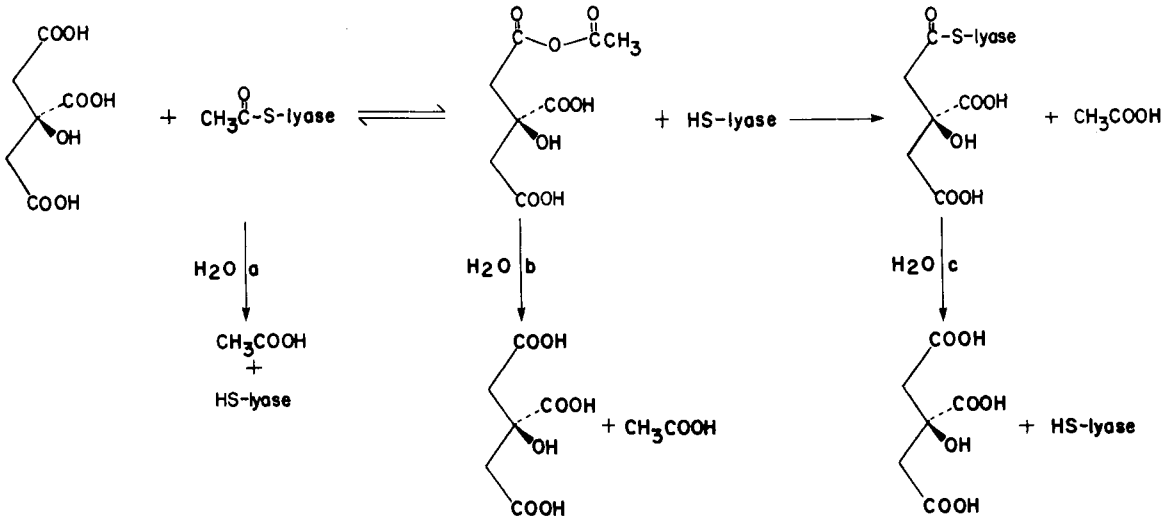
Table II: Turnover-Dependent Inactivation of Citrate Lyase[a]

| compd | metal (2 mM) | partition ratio | $k_{inact}$ ($min^{-1}$) |
|---|---|---|---|
| citrate | Mg(II) | 1500 | 0.73 |
| | Zn(II) | 6500–12000 | 0.063–0.12 |
| (−)-*erythro*-2-fluorocitrate | Mg(II) | 18 | 0.14 |
| | Zn(II) | 23 | 0.27 |
| (+)-*erythro*-2-fluorocitrate | Mg(II) | >105 | <0.006 |
| | Zn(II) | >95 | <0.004 |
| (−)-*erythro*-2-hydroxycitrate | Mg(II) | 15 | 0.067 |
| | Zn(II) | 38 | 0.032 |
| (+)-*erythro*-2-hydroxycitrate | Mg(II) | <0.1 | 1.1 |
| | Zn(II) | $<10^{-4}$ | 0.68 |
| (−)-*threo*-2-hydroxycitrate | Mg(II) | >90 | <0.005 |
| | Zn(II) | >70 | <0.005 |
| (+)-*threo*-2-hydroxycitrate | Mg(II) | 55 | 0.042 |
| | Zn(II) | <1 | 0.090–0.13 |

[a] The partition ratios and $k_{inact}$ values were determined under the same conditions used to determine the $k_{cat}$ values. A $k_{inact}$ value for an isomer processed by citrate lyase is equal to the $k_{cat}$ value divided by the partition ratio. The partition ratio is equal to the moles of substrate cleaved (1:1 correspondence to moles of NADH oxidized) divided by the moles of functional active site present. The partition ratios listed as upper and lower limits are based on the detection limits of either $k_{cat}$ or $k_{inact}$, respectively. A $k_{inact}$ value for a derivative that is not cleaved was calculated from the time-dependent loss of citrate lyase activity when incubated with the particular derivative (see Materials and Methods).

These rates were initially measured in the presence of $Zn^{2+}$ because this metal minimizes the turnover-dependent inactivation of citrate lyase by citrate (Singh & Srere, 1971). This type of inactivation is illustrated in eq 3, and the results are shown on the top of Table II. These activity assays were repeated in the presence of $Mg^{2+}$, the divalent metal ion used most frequently in the literature. In the presence of $Mg^{2+}$, citrate turnover by citrate lyase is at a maximum (see Table I) (Singh & Srere, 1971). The cleavage rate of the (−)-*erythro*-fluoro isomer, unlike that for citrate, is 2.4 times faster in the presence of $Zn^{2+}$ than in $Mg^{2+}$. This altered order of

Scheme II



reactivity is unique to this 2-fluorocitrate stereoisomer and not yet understood.

*Catalytic Cleavage of the 2-Hydroxycitrate Isomers.* Previous studies showed that three of the four 2-hydroxycitrate stereoisomers are catalytically cleaved by citrate lyase in the presence of 10 mM $Mg^{2+}$ (Sullivan et al., 1977a). We have repeated these measurements in the presence of 2 mM $Zn^{2+}$ to directly compare the properties of a fluorine or hydroxyl-group substitution at the C-2 of citrate. Concentrations of zinc higher than 2 mM seemed to inhibit turnover and precipitate the 2-hydroxycitrates. Also, to correlate the effect of $Zn^{2+}$ or $Mg^{2+}$ on the turnover of the 2-hydroxycitrates, $k_{cat}$ values were redetermined for these citrate analogues in the presence of 2 mM $Mg^{2+}$.

The rates of 2-hydroxycitrate turnover (Table I) in the presence of 2 mM $Mg^{2+}$ exhibit the same relative reactivity as they did in 10 mM $Mg^{2+}$ (Sullivan et al., 1977a): (+)-threo > (−)-erythro > (−)-threo. No activity was detected with the (+)-erythro isomer in the presence of 2 or 10 mM $Mg^{2+}$, nor was any detected when $Zn^{2+}$ was present. Substitution of $Zn^{2+}$ for $Mg^{2+}$ affects the turnover of (2*R*,3*S*)-(−)-*erythro*- and (2*S*,3*S*)-(−)-*threo*-2-hydroxycitrate less than it does for either 2-fluorocitrate or citrate. The extreme metal specificity of (+)-*threo*-2-hydroxycitrate turnover is unique. This isomer is processed at a faster rate (2.3 $min^{-1}$) than any other 2-hydroxycitrate if $Mg^{2+}$ is present; if $Zn^{2+}$ is present, no rate is detectable. In addition, 1 mM $Zn^{2+}$ will completely inhibit the turnover of this isomer in the presence of 1 mM $Mg^{2+}$.

Although a fluorine substituent may often mimic certain attributes of a hydroxyl group [Rokita et al. (1982) and references cited therein], the net result is not always predictable. (−)-*erythro*-2-Hydroxycitrate and its stereochemical equivalent, (−)-*erythro*-2-fluorocitrate, behave very differently. Their cleavage rates in the presence of $Zn^{2+}$ differ by 5-fold and only the fluoro isomer is highly sensitive to the metal ion present. The contrasting reactivities of fluoro vs. hydroxy stereoequivalents are even more apparent with the (+)-erythro isomers. Citrate lyase has demonstrated a tolerance to polar substituents vicinal to bond cleavage by catalytically processing (+)-*erythro*-2-fluorocitrate. The corresponding (+)-*erythro*-2-hydroxycitrate, however, is not processed by this enzyme for reasons outlined below.

*Turnover Inactivation of Citrate Lyase with the 2-Fluorocitrate Stereoisomers.* Citrate lyase has already proven to be unstable during citrate turnover as outlined in eq. 3. The partitioning between catalytic turnover and inactivation can be attributed to the adventitious hydrolysis of acyl intermediates produced by the subunit of citrate lyase responsible for the transacylation activity (Dimroth & Eggerer, 1975a). By studying the partitioning of 2-fluorocitrate turnover, one can assess the influence of the fluorine on the chemical stability of the catalytic intermediates.

For citrate-induced inactivation, HS-lyase could result from (a) the hydrolysis of the resting enzyme (not seen), (b) hydrolysis of the intermediate during the transacylation reaction, a mixed anhydride of acetic and citric acids, or (c) hydrolysis of the product from the transacylation step, a citrylated citrate lyase (citryl-S-lyase) (Scheme II). Citryl-S-lyase has not yet been isolated, nor has its hydrolysis rate been determined because, once it forms, it quickly reacts to complete the catalytic cycle. However, its stability may be similar to that of a noncleavable citrate analogue, 3-fluoro-3-deoxycitrate (Rokita et al., 1982). This forms a relatively stable acyl lyase complex, 3-fluoro-3-deoxycitryl-S-lyase, which hydrolyzes with a $t_{1/2}$ of ca. 70 min. The citrate-induced inactivation of citrate lyase may also be modeled by the action of 3-fluoro-3-deoxycitrate. The acetic 3-fluoro-3-deoxycitric mixed anhydride is not stable, and its hydrolysis accounts for a net hydrolysis of acetyl-S-lyase to the inactive HS-lyase.

If the corresponding anhydride intermediates of 2-fluorocitrate processing are more sensitive to hydrolysis than those for citrate, then their partition ratios could be lower (fewer turnovers per inactivation) than that for citrate. Unfortunately, the general instability of citrate lyase in solution (Srere, 1975) limits the detection of substrate-dependent inactivation. The large partition ratio of citrate processing is easily determined because many catalytic cycles per enzyme can be observed in a short period (7500 cycles in 10 min). If only 100 cycles were monitored, no inactivation could be detected. With (+)-*erythro*-2-fluorocitrate (Table II), 63 cycles can be monitored in 100 min with little inactivation, suggesting a maximum $k_{inact}$ of 0.006 $min^{-1}$ in the presence of $Mg^{2+}$. Therefore, no hydrolysis of either the mixed anhydride of acetic and (+)-*erythro*-2-fluorocitric acids or the 2-fluorocitryl-S-lyase was evident. The (+)-erythro isomer may inactivate citrate lyase more efficiently than citrate (when comparing partition ratios); but under the experimental conditions, this was not detected.

From the $K_I$ determination for (−)-*erythro*-2-fluorocitrate in the time-dependent inactivation assays noted above, a $k_{inact}$ was calculated to be 0.13 $min^{-1}$. This compares favorably to a $k_{inact}$ of 0.027 $min^{-1}$ calculated from the partition ratio of $k_{cat}$ to molecules of product formed before total catalytic in-

activation (Table II). The turnover-dependent inactivation of citrate lyase demonstrated here appears to result from a net deacetylation of the active enzyme, acetyl-S-lyase, to an inactive form bearing the free thiol, HS-lyase. Reacetylation and therefore reactivation by acetic anhydride is diagnostic of this type of inactivation (Buckel et al., 1971; Srere et al., 1972). As expected, the catalytic activity of citrate lyase inactivated by (−)-*erythro*-2-fluorocitrate can be recovered by the addition of acetic anhydride. Essentially complete inactivation of citrate lyase occurs after 30 min at 25 °C in the presence of 1.5 mM (−)-*erythro*-2-fluorocitrate, but 33% of the initial activity can be restored by acetic anhydride. Deacetylated citrate lyase, HS-lyase, produced by other techniques has been reactivated with acetic anhydride yielding up to 50% of the initial activity (Buckel et al., 1971; Srere et al., 1972; Rokita et al., 1982).

(−)-*erythro*-2-Fluorocitrate inactivates citrate lyase at least 500 times more efficiently than citrate, as indicated by their partition ratios of ca. 20 and ca. 10 000, respectively. This varying ability of citrate or its fluorinated analogues to inactivate citrate lyase may reflect the hydrolysis rate of the appropriate mixed anhydride and the rates for transfer of either acyl moiety back to the enzyme. Due to the stereospecificity of enzyme cleavage, there is another possible mechanism by which (−)-*erythro*- but not (+)-*erythro*-2-fluorocitrate could inactivate citrate lyase. Catalytic turnover of this isomer leaves the enzyme in the active, but modified, form of fluoroacetyl-S-lyase (instead of acetyl-S-lyase). If fluoroacetyl-S-lyase or the mixed anhydride of fluorocitric and fluoroacetic acid (in subsequent catalytic cycles) is very sensitive to hydrolysis, the partition ratio will drop. A more facile deacetylation of fluoroacetyl-S-lyase vs. acetyl-S-lyase (or the mixed anhydride mentioned above) could be expected since fluoroacetic acid has a $pK_a$ (=2.6) 2 $pK_a$ units lower than acetic acid ($pK_a$ = 4.7) (Streitwieser & Heathcock, 1976).

The transacylation step, when catalyzed by the appropriate citrate lyase subunit, is not dependent on the presence of metals (Dimroth & Eggerer, 1975a). However, the $k_{inact}$ values reported here and elsewhere (Singh & Srere, 1971; Sullivan et al., 1977a) for the native enzyme do vary with the metal present; this may reflect a proposed metal-dependent conformational change of the enzyme (Sivaraman & Sivaraman, 1979). There is no clear explanation of why the effect of magnesium and zinc varies with each substrate.

One advantage of measuring the partition ratio (of turnover vs. inactivation) is that this term clearly defines the average number of successful catalytic cycles before enzyme inactivation occurs. For every one fluorinated substrate analogue–enzyme encounter that results in enzyme inactivation, at least 18 encounters initiate and successfully complete a catalytic cycle. Therefore, the majority of these encounters mimic the productive encounter of citrate and the enzyme.

*Turnover Inactivation of Citrate Lyase with the 2-Hydroxycitrate Stereoisomers.* Sullivan et al. (1977a) have published $k_{inact}$ values for the time-dependent inactivation of citrate lyase by the four 2-hydroxycitrate stereoisomers. We have repeated this work, and we have calculated the $k_{inact}$ values by measuring the partition ratios for reasons mentioned above. In addition, we have lowered the divalent metal ion concentration ($Mg^{2+}$ and $Zn^{2+}$) of the assays to 2 mM in order to make direct comparison to the data for the 2-fluorocitrate stereoisomers. Since the partition ratios depend on enzyme turnover, the citrate lyase interactions with (+)-*threo*-2-hydroxycitrate ($Zn^{2+}$) and (+)-*erythro*-2-hydroxycitrate ($Zn^{2+}$ and $Mg^{2+}$) are discussed in the following section. The inactivation rates presented here (Table II) are in good agreement with those determined by Sullivan et al. (1977a, see p 7585). Turnover of (2*R*,3*S*)-(−)-*erythro*- and (2*R*,3*R*)-(+)-*threo*-2-hydroxycitrate inactivates citrate lyase with roughly the same efficiency (less than 60 turnovers per inactivation) as the (−)-*erythro*-2-fluorocitrate (2*R*,3*R*). In all cases, inactive enzyme will regain at least 33% of its original activity in the presence of acetic anhydride. The (−)-*threo*-2-hydroxycitrate (2*S*,3*S*) may inactivate citrate lyase; but with the sensitivity of the assays employed, the $k_{inact}$ value must be less than 0.005 $min^{-1}$.

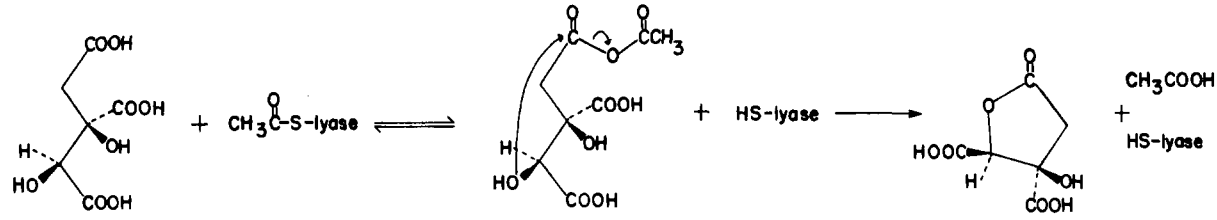
Because there is a plethora of tridentate 2-hydroxycitrate chelation patterns, Sullivan et al. (1977a) and Stallings et al. (1979) explained the inactivation of citrate lyase by the 2-hydroxycitrate stereoisomers as a function of their citrate-type and non-citrate-type binding to the active site. The role of nonproductive binding, however, is not necessary to explain substrate-dependent inactivation of citrate lyase to date. The inactivation of citrate lyase by the 2-hydroxycitrates can result from the production of unstable catalytic intermediates produced during turnover.

The low partition ratios of the 2-hydroxycitrate isomers most likely result from inactivation mechanisms similar to those described for (−)-*erythro*-2-fluorocitrate. A mixed anhydride and an acyl-enzyme intermediate form during turnover; at least one of these species is more sensitive to hydrolysis than the corresponding species produced during citrate turnover. The variation between the partition ratios (15 vs. <90 with $Mg^{2+}$) for the 2-hydroxycitrate stereoisomers may reflect the differing rates of hydrolysis (as compared to the rates of continued turnover) for the catalytic intermediates with different regio- and stereochemistry. Since the (−)-*erythro*- and (−)-*threo*-2-hydroxycitrate isomers cleave to yield glycolyl-S-lyase, their partition ratios could also reflect the hydrolysis of the glycolyl moiety. Glycolic acid, like fluoroacetic acid, has a lower $pK_a$ (=3.8; Streitwieser & Heathcock, 1976) than acetic acid, which could facilitate the thiol ester hydrolysis of a resting acyl enzyme. However, the presence of a glycolyl moiety per se cannot explain the low partition ratio of the (−)-erythro isomer because both the (−)-erythro and the (−)-threo isomers form glycolyl-S-lyase and only the (−)-erythro isomer has a detectable partition ratio. This result suggests in turn that the hydrolysis of fluoroacetyl-S-lyase might not contribute to the low partition ratio of (−)-*erythro*-2-fluorocitrate to any great extent. Since each acyl-S-lyase characterized to date (this paper; Rokita et al., 1982) is relatively stable, most enzyme inactivation must arise from the hydrolysis of the mixed anhydride (Scheme II, reaction b).

*Citrate Lyase Inactivation with (+)-erythro- and (+)-threo-2-Hydroxycitrate.* The $k_{inact}$ values of the (+)-*threo*-hydroxy isomer with $Zn^{2+}$ and the (+)-*erythro*-hydroxy isomer with either $Zn^{2+}$ or $Mg^{2+}$ could not be calculated from partition ratios because neither was catalytically cleaved by citrate lyase under these conditions. We have repeated the measurements of Sullivan et al. (1977a) to determine the $k_{inact}$ values for these analogues in time-dependent inactivation studies, but we have substituted previous assay conditions with those used consistently in this paper. We found the $k_{inact}$ of (+)-*erythro*-2-hydroxycitrate, a naturally occurring hydroxycitrate (Table II), to be larger than the $k_{inact}$ for all of the other hydroxy analogues, in good agreement with Sullivan et al. (1977a, see p 7585).

Citrate lyase inactivated by either of these two 2-hydroxycitrates yields deacetylated HS-lyase, and as expected, this inactive enzyme can be reactivated to ca. 50% of its original

Scheme III




activity after treatment with acetic anhydride. These analogues may not be processed by citrate lyase because of the added steric hindrance, but this would not explain their ability to deacetylate the enzyme. Sullivan et al. (1977a) suggested that this ability arose from nonproductive binding to the active site, allowing for the hydrolysis of the necessary acetyl group from acetyl-S-lyase. However, the activities of these hydroxy isomers with citrate lyase can still be explained by normal, citrate-type binding and by the proposed catalytic mechanism of this enzyme (eq 1 and 2). The lack of detectable substrate cleavage may reflect only a competition between a very slow turnover rate and a facile inactivation step during turnover—hydrolysis of an acyl intermediate. The $k_{inact}$ of the (+)-*threo*-hydroxy isomer (Table II) is 0.090–0.13 $min^{-1}$ ($Zn^{2+}$), only ca. 3 times slower than the slowest $k_{cat}$ (Table I). The $k_{inact}$ values for (+)-*erythro*-2-hydroxycitrate (0.68–1.1 $min^{-1}$), however, are larger than the $k_{cat}$ values for its fluorinated stereoequivalent, (+)-*erythro*-2-fluorocitrate.

Scheme III suggests a possible mechanism in which the (+)-*erythro*-hydroxy isomer (but not the corresponding fluorocitrate) may deacetylate the enzyme without completing the catalytic cycle. Every enzyme turnover initiated would result in enzyme inactivation (a partition ratio of 0 products formed per inactivation) due to this very efficient mechanism for hydrolysis. If an acetic hydroxycitric mixed anhydride is formed with the—by now anticipated—regiospecificity of citrate turnover, the hydroxyl group in the corresponding *pro-R* arm might react intramolecularly to cyclize the substrate (forming a stable hydroxycitrate lactone and acetate) and generate inactive HS-lyase. This type of argument has been advanced to explain the larger uncoupling of the ATPase vs. carbon-bond cleavage activities of ATP citrate lyase for the 2-hydroxycitrates containing *pro-R*, instead of *pro-S*, hydroxyl groups (Rokita et al., 1982) and to explain the lack of citrate synthase activity with the 2-hydroxycitrates (Sullivan et al., 1977a). The facilitated hydrolysis illustrated above could occur at the stage of either the mixed anhydride (Scheme II, reaction b) or the acyl enzyme (2-hydroxycitryl-S-lyase). The mixed anhydride hydrolysis is shown since this intermediate seems most sensitive to hydrolysis during the cleavage of other substrates. This intramolecular inactivation pathway is not available to the corresponding fluorinated analogue and, thus, could explain a major difference in the enzymic fates of a 2-fluoro- or 2-hydroxy-substituted citrate [e.g., (+)-erythro isomers]. Although a fluorine substitution can often mimic the inductive effects of a hydroxyl group, its nucleophilicity is much less than that of a hydroxyl group.

(+)-*threo*-2-Hydroxycitrate (2*R*,3*R*), also with a corresponding *pro-R* hydroxyl group, can be catalytically cleaved only in the presence of $Mg^{2+}$, but it is an effective inactivator of citrate lyase with either metal present. The active site geometry of citrate lyase might selectively inhibit lactonization of the (+)-threo isomer ($k_{inact}$ = 0.042 $min^{-1}$ with $Mg^{2+}$) during turnover compared to the lactonization of the (+)-*erythro*-hydroxy isomer ($k_{inact}$ = 1.1 $min^{-1}$). However, the inhibitory effect of the (+)-threo analogue increases ca. 10-fold in the presence of $Zn^{2+}$. The metal substitution increases the efficiency of enzyme inactivation (lower partition ratio) by both lowering the $k_{cat}$ value and increasing the $k_{inact}$ value to a competitive level.

Three enzymes, citrate synthase, mammalian ATP citrate lyase, and bacterial citrate lyase, are known to catalyze the cleavage and formation of citrate via the reversible aldol-type condensation of oxalacetate and an acetate derivative. Previously, two of these enzyme were shown to process both citrate and its fluorinated analogues with the same regiospecificity [for citrate synthase, see Fanshier et al. (1964); for ATP citrate lyase, see Marletta et al. (1981)]. This paper demonstrates that the third enzyme, bacterial citrate lyase, acts with the same regiospecificity whether it catalyzes the cleavage of citrate or the 2-fluoro- or 2-hydroxycitrates. Both the fluoro- and hydroxyl-group substituents dramatically slow the carbon–carbon bond cleavage process whether they are geminal or vicinal to the bond cleaved.

The fluorine and hydroxy derivatives of citrate alter the normal catalytic flux of intermediates for the bacterial citrate lyase as well as for the mammalian ATP citrate lyase (Rokita et al., 1982). The consequences of citrate analogue turnover are dictated by the contrasting mechanisms proposed for these enzymes. In the presence of 2-fluoro- or 2-hydroxycitrates, ATP citrate lyase catalyzes a futile ATPase cycle between 4 and 500 times faster than carbon–carbon bond cleavage. These analogues do not inactivate this mammalian enzyme (Rokita et al., 1982). On the other hand, during the catalytic cycle of citrate lyase, unstable intermediates (most likely the mixed anhydrides) are produced that cause enzyme inactivation not by covalent enzyme modification but rather by ultimately producing the deacetylated form of citate lyase. The rate of inactivation, concomitant with the hydrolysis of the acyl intermediates, depends on the chemical composition and stereochemistry of these intermediates but not necessarily on the chelation patterns of the citrate derivatives. (+)-*erythro*-2-Hydroxycitrate is the most efficient inactivator (lowest partition ratio, largest $k_{inact}$); in fact, no turnover products can be detected, only inactivation. Other citrate derivatives, (−)-*erythro*- and (+)-*threo*-2-hydroxycitrates and (−)-*erythro*-2-fluorocitrate (the toxic 2-fluorocitrate) catalyze the inactivation of citrate lyase less efficiently, allowing 15–55 turnovers per inactivation ($Mg^{2+}$). Neither (+)-*erythro*-2-fluorocitrate nor (−)-*threo*-2-hydroxycitrate (epimeric equivalents) detectably inactivate citrate lyase, perhaps because they are processed as very slow substrates. Finally, if the deacetylated form of citrate lyase can be effectively reacetylated in vivo by acetyl coenzyme A and the loading enzyme (Dimroth et al., 1977), none of these 2-fluoro- or 2-hydroxycitrates may be in vivo inactivators.

Acknowledgments

We thank Dr. A. Sullivan, Hoffmann-La Roche Inc., for the 2-hydroxycitrate stereoisomers and Dr. M. Marletta, Department of Nutrition and Food Science, Massachusetts Institute of Technology, for the 2-fluorocitrates.

**Registry No.** Citrate lyase, 9012-83-3; citric acid, 77-92-9; (−)-*erythro*-2-fluorocitric acid, 31654-44-1; (+)-*erythro*-2-fluorocitric acid, 74841-44-4; (−)-*erythro*-2-hydroxycitric acid, 6205-15-8; (+)-*erythro*-2-hydroxycitric acid, 27750-11-4; (−)-*threo*-2-hydroxycitric acid, 27750-10-3; (+)-*threo*-2-hydroxycitric acid, 6385-10-0.

References


Bergmeyer, H., Ed. (1974) *Methods of Enzymatic Analysis*, p 442, Academic Press, New York.

Bowen, T. J., & Mortimer, M. G. (1971) *Eur. J. Biochem. 23*, 262.

Buckel, W., Buschmeier, V., & Eggerer, H. (1971) *Hoppe-Seyler's Z. Physiol. Chem. 352*, 1195.

Carrell, H. L., Glusker, J. P., Villafranca, J. J., Mildvan, A. S., Dummel, R. J., & Kun, E. (1970) *Science (Washington, D.C.) 170*, 1412.

Dimroth, A., & Eggerer, H. (1975a) *Proc. Natl. Acad. Sci. U.S.A. 72*, 3458.

Dimroth, A., & Eggerer, H. (1975b) *Eur. J. Biochem. 53*, 227.

Dimroth, A., Loyal, R., & Eggerer, H. (1977) *Eur. J. Biochem. 80*, 479.

Dummel, R. J., & Kun, E. (1969) *J. Biol. Chem. 244*, 2966.

Fanshier, D. W., Gottwald, L. K., & Kun, E. (1964) *J. Biol. Chem. 239*, 425.

Filler, R., Ed. (1976) *ACS Symp. Ser. No. 28.*

Glusker, J. P. (1971) *Enzymes, 3rd Ed. 5*, 413.

Glusker, J. P. (1980) *Acc. Chem. Res. 13*, 345.

Glusker, J. P., & Srere, P. A. (1973) *Bioorg. Chem. 2*, 301.

Goldstein, J. A., Cheng, Y., Marletta, M. A., & Walsh, C. (1978) *Biochemistry 17*, 5567.

Lewis, Y. S., & Neelakantan, S. (1965) *Phytochemistry 4*, 619.

Marletta, M. A., Srere, P. A., & Walsh, C. T. (1981) *Biochemistry 20*, 3719.

Oppenheimer, N. J., Arnold, L. J., & Kaplan, N. O. (1971) *Proc. Natl. Acad. Sci. U.S.A. 68*, 3200.

Peters, R. (1972) *Carbon-Fluorine Compounds: Chemistry, Biochemistry and Biological Activities, a Ciba Foundation Symposium*, London, September 13–15, 1971, Associated Scientific Publishers, Amsterdam.

Rokita, S. E., Srere, P. A., & Walsh, C. T. (1982) *Biochemistry 21*, 3765.

Singh, M., & Srere, P. A. (1971) *J. Biol. Chem. 246*, 3847.

Singh, M., Dempsey, W. B., & Srere, P. A. (1975) *J. Bacteriol. 124*, 686.

Sivaraman, H., & Sivaraman, C. (1979) *FEBS Lett. 105*, 267.

Srere, P. A. (1975) *Adv. Enzymol. Relat. Areas Mol. Biol. 43*, 57.

Srere, P. A., Bottger, B., & Brooks, G. C. (1972) *Proc. Natl. Acad. Sci. U.S.A. 69*, 1201.

Stallings, W. C., Blount, J. F., Srere, P. A., & Glusker, J. P. (1979) *Arch. Biochem. Biophys. 193*, 431.

Stallings, W. C., Monti, C. T., Belvedere, J. F., Preston, R. K., & Glusker, J. P. (1980) *Arch. Biochem. Biophys. 203*, 65.

Streitwieser, A., & Heathcock, C. H. (1976) *Introduction to Organic Chemistry*, p 429, MacMillan, New York.

Sullivan, A., Singh, M., Srere, P. A., & Glusker, J. P. (1977a) *J. Biol. Chem. 252*, 7583.

Sullivan, A., Triscari, J., & Spiegel, H. E. (1977b) *Am. J. Clin. Nutr. 30*, 777.

Walsh, C. T. (1983) *Adv. Enzymol. Relat. Areas Mol. Biol.* (in press).


# Chiral Instability at Sulfur of *S*-Adenosylmethionine†

Su-Er Wu, William P. Huskey,‡ Ronald T. Borchardt, and Richard L. Schowen*

ABSTRACT: *S*-Adenosylmethionine, generated enzymically in chirally pure form (*S* configuration at sulfur), undergoes simultaneous irreversible conversion to 5′-deoxy-5′-(methylthio)adenosine and homoserine with a rate constant of $6 \times 10^{-6}$ $s^{-1}$ at pH 7.5 and 37 °C and reversible conversion to an enzymically inactive stereoisomer (*R* configuration at sulfur) with a forward rate constant of $8 \times 10^{-6}$ $s^{-1}$ at pH 7.5 and 37 °C. These forms of instability require small turnover times and/or stabilization through macromolecular binding for *S*-adenosylmethionine, if organisms that utilize it are to avoid losses of metabolic energy.

The compound *S*-adenosylmethionine (AdoMet[1]), the methyl donor for numerous enzymic transmethylation reactions which play the most diverse roles in biochemistry (Cantoni, 1952, 1953, 1960; Usdin et al., 1979, 1981), is structurally complex. This molecular complexity is thought to be critical for enzyme–substrate interactions in the catalytic transition state, which lead to the large catalytic accelerations that transmethylases achieve [$10^{16}$ for catechol *O*-methyltransferase (COMT); Olsen et al., 1979]. On the other hand, the elaborate structure is paid for by the organism through the very high energy cost of AdoMet biosynthesis (Cantoni, 1952, 1960). Atkinson (1977) writes "... in being reduced and activated to form the methyl group of *S*-adenosylmethionine, a carbon atom from glucose is promoted from an average value of 6.3 [ATP] equivalents (38/6) to a cost of 12 equivalents. Active methyl is probably the most expensive metabolic compound or group on a per-carbon basis". This suggests that organisms should experience a strong evolutionary pressure toward preservation and efficient utilization of AdoMet.

† From the Departments of Biochemistry and Chemistry and the Center for Biomedical Research, University of Kansas, Lawrence, Kansas 66045. *Received August 6, 1982.* This research was supported by grants from the National Institutes of Health (GM20199 and GM29332).

‡ National Science Foundation Graduate Fellow.

[1] Abbreviations: AdoHcy, *S*-adenosyl-L-homocysteine; AdoMet, *S*-adenosylmethionine; AdoMet synthetase, *S*-adenosylmethionine synthetase; ATP, adenosine 5′-triphosphate; COMT, catechol *O*-methyltransferase; DHA, 3,4-dihydroxyacetophenone; DTT, dithiothreitol; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; InoHcy, *S*-inosyl-L-homocysteine; MTA, 5′-deoxy-5′-(methylthio)adenosine; SA, specific activity; HPLC, high-pressure liquid chromatography.

0006-2960/83/0422-2828$01.50/0 