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Inhibition of Liver Aconitase Isozymes by (—)-*erythro*-Fluorocitrate

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SUMMARY

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A highly purified cytosol aconitase [citrate (isocitrate) hydrolyase, EC 4.2.1.3] was isolated from pig liver. Whereas purified aconitase preparations previously reported in the literature require cysteine and Fe^{2+} for maximal catalytic activity, the purified cytosol enzyme and the partially purified mitochondrial aconitase described here need no added activator. The molecular weight of the purified cytosol enzyme as determined by sucrose density gradient centrifugation was between 107,000 and 111,000. Inhibitory effects of (−)-*erythro*-fluorocitrate on both aconitase isoenzymes were studied in the absence and presence of Mg^{2+} and Mn^{2+}. Fluorocitrate was a competitive reversible inhibitor of both aconitases, as deduced from steady-state kinetic analyses (average K_i = 22–45 μM). The bivalent cations Mg^{2+} and especially Mn^{2+}, when incubated with either aconitase isoenzyme, inhibited enzymatic activity, an effect which increased with time. A high concentration (30 mM) of citrate reversed the inhibition and protected the enzyme against inhibition by Mg^{2+} or Mn^{2+}. When (−)-*erythro*-fluorocitrate and Mg^{2+} (or Mn^{2+}) were present simultaneously, as in the isocitrate dehydrogenase coupled assay system, the time-dependent inhibition which ensued was a result of competitive inhibition by fluorocitrate and the more complex inhibition of the enzyme by Mg^{2+} or Mn^{2+}

INTRODUCTION

Inhibition of aconitase [citrate (isocitrate) hydrolyase, EC 4.2.1.3] by fluorocitrate has been generally considered to be the enzymatic basis of fluorocitrate poisoning (1, 2). More critical analysis of published experiments reveals that the majority of work concerned with the inhibition of aconitase by fluorocitrate has been carried out with fluorocitrate preparations of varying degrees of purity and with aconitase containing tissue extracts which were relatively poorly defined (3). It is well known that purified aconitase (4) is unstable, and until recently (5, 6)

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only a small degree of purification of this enzyme has been achieved by most investigators. In view of these uncertainties, reported K_i values for fluorocitrate and the types of inhibitions observed varied considerably (see ref. 3). Enzymatically synthesized fluorocitrate from fluoroacetyl-CoA and oxalacetate inhibited crude aconitase extracted from kidney mitochondria in an apparently nonlinear competitive manner, with K_i in the micromolar range (7). A linear competitive K_i value of 290 μM was obtained with synthetic, resolved (−)-*erythro*-fluorocitrate (8) and homogeneous aconitase of pig heart by Villafranca and Mildvan (5) (see ref. 6). In sharp contrast to these relatively similar values, Brand *et al.* (9) reported that (−)-*erythro*-fluorocitrate inhibited crude aconitase obtained by solubilization of liver mitochondria in a partially competitive manner, with a K_i of 34 nM, when citrate was the substrate, and the inhibition was partially noncompetitive when *cis*-aconitate was the substrate. It would appear that the lethal effect of fluorocitrate could be more readily explained by K_i values in the 10 nM range and by a noncompetitive mechanism, whereas reversible competitive inhibition (6, 7) tends to argue against the assumption that aconitase is the main cellular target site of fluorocitrate.

Interpretation of the molecular toxicological mechanism of action of fluorocitrate, in terms of aconitase inhibition, is complicated by several factors. We as well as others (6, 7) observed that fluorocitrate exhibited an apparent time-dependent inhibitory effect on aconitase when this enzyme was assayed in the presence of Mg^{2+} or Mn^{2+} and isocitrate dehydrogenase (EC 1.1.1.42) and the reaction was monitored by the reduction of $NADP^+$. This effect was absent when aconitase was assayed according to Racker (10) by measuring the rate of *cis*-aconitate formation at 240 nm. A further complication arises from the discovery that there are two distinct aconitase isozymes in most animal cells (11–14). Thus it would be desirable to compare the inhibitory effects of fluorocitrate on both enzymes of the same tissue before a kinetic interpretation of the cellular toxicity of fluorocitrate can be formulated. We have shown recently that the cytoplasmic and mitochondrial aconitase isozymes of pig tissues greatly differ in their isoelectric points and stabilities (15).

As an attempt to clarify the mode of action of (−)-*erythro*-fluorocitrate on liver aconitase isozymes, we isolated the cytoplasmic enzyme at a purity comparable to the homogeneous heart aconitase (5, 6) and also obtained a partially purified mitochondrial isozyme from the same tissue. Since previously observed anomalous inhibition by (−)-*erythro*-fluorocitrate occurred only in the presence of bivalent cations required for the isocitrate dehydrogenase coupled test system, the actions of Mg^{2+} and Mn^{2+} were also studied in the presence and absence of fluorocitrate. Under steady-state conditions (−)-*erythro*-fluorocitrate exhibited a strictly competitive and reversible inhibitory effect on both aconitase isozymes. This inhibition was unaffected by Mg^{2+}, provided that kinetic analyses were based on initial reaction velocities. On the other hand, Mg^{2+} and especially Mn^{2+} exerted a time-dependent, nonlinear inhibitory effect on aconitase, which greatly exaggerated inhibition by fluorocitrate, when both either cation and fluorocitrate were present simultaneously in the coupled assay system.

From an experimental point of view it was important to obtain aconitase isozymes which require for maximal activity no Fe^{2+}-cysteine activator, generally needed for maximal activity of the pure enzyme (5, 6). This was necessary because the association constants of Mg^{2+}-citrate and Mg^{2+}-fluorocitrate cannot be determined in the presence of any other bivalent cation which could be chelated by carboxylate substrates. Determination of Mg^{2+}-citrate and Mg^{2+}-fluorocitrate affinity constants were necessary in order to calculate K_m and K_i values in the presence of Mg^{2+}.

EXPERIMENTAL PROCEDURE

Materials

Citric, *dl*-isocitric, and *cis*-aconitic acids, Tris base (containing less than 1 ppm of heavy metal as controlled by atomic absorption), and alcohol and isocitrate dehydrogenases were products of Calbiochem. Resins and molecular sieves were obtained

from Bio-Rad. *N*,*N*′-Methylenebis(acrylamide), acrylamide, and *N*,*N*,*N*′,*N*′-tetramethylenediamine were purchased from Eastman Organic Chemicals; ammonium persulfate, from Mallinckrodt; Coomassie blue, from Sigma; ultrapure sucrose, from Mann, phenazine methosulfate and 2-(*p*-iodophenyl)-3-(*p*-nitrophenyl)-5-phenyltetrazolium chloride, from Aldrich Chemical Company; and hemoglobin, from Nutritional Biochemicals. Double-distilled water was used for all solutions. Optically resolved (−)-*erythro*-fluorocitrate was synthesized as published (8). The X-ray diffraction pattern of synthetic optically resolved fluorocitrate has been determined (16, 17).

Methods

Aconitase was assayed spectrophotometrically (10) during purification by following the rate of *cis*-aconitate formation at 240 nm from 30 mM citrate brought to pH 7.5 (25°) with Tris base. The millimolar absorption coefficient of 3.414 (18) was used for calculation of the rates of *cis*-aconitate formation. When aconitase was coupled to isocitrate dehydrogenase, the test system contained 1 mM citrate, 0.26 mM $NADP^+$, and 0.1 mg/ml of aconitase-free isocitrate dehydrogenase. In this system the rates of NADPH formation were measured spectrophotometrically at 340 nm in 2 mM Tris-HCl pH 7.4. The reaction was started with aconitase, and initial rates were measured within 15–30 sec. For enzyme assays, a Zeiss PMQ II spectrophotometer was connected to a recorder and a scale expansion unit (1:5), allowing a full-scale reading of 0.040 absorbance with cuvettes of 5-cm light path. Either Mn^{2+} or Mg^{2+} was the activator of isocitrate dehydrogenase, as described in detail under RESULTS. Alcohol dehydrogenase was assayed at pH 8.5 (Tris-HCl, 50 mM) in the presence of 170 mM ethanol and 4 mM NAD^+. Calculations of K_i and K_m values were based on double-reciprocal plots, obtained by least-squares fits designed for a PDP-12 digital computer (Digital Equipment Corporation). Association constants for citrate-Mg^{2+} and (−)-*erythro*-fluorocitrate–Mg^{2+} complexes were determined by an ion-exchange method (19). The PDP-12 computer was also used for the calculation of free substrate concentrations in the presence of added bivalent cations. Since Tris has no significant binding affinity toward Mg^{2+} (20), this buffer was used in all experiments. The concentration of Mg^{2+} was determined by atomic absorption photometry in a Perkin-Elmer 403 instrument.

Polyacrylamide gel electrophoresis (21) was carried out in 6% polyacrylamide gel prepared in 50 mM Tris–1 mM citrate (pH 8.3) at 0° and conducted for 3 hr at a potential span of 300 V at 0–4°. At the end of the run the discs were sliced sagitally. Half of each disc was stained for protein with 0.02% Coomassie blue in 12.5% trichloracetic acid. The other half was stained for aconitase activity by incubation for 1 hr at 25° in a medium containing 50 mM Tris-HCl (pH 7.5), 10 mM *cis*-aconitate, 5 mM $MnCl_2$, 0.8 mM *p*-iodotetrazolium violet, 0.04 mM phenazine methosulfate, 0.8 mM $NADP^+$, and 8 mg/ml of isocitrate dehydrogenase. Molecular weight was determined in a linear sucrose gradient (22) between 0.146 and 0.584 M at pH 7.5 in 50 mM Tris-HCl according to published techniques (23). The Spinco model L-2 centrifuge with SW 50-L swinging bucket rotor was run at 0° for 14 hr at 105,000 × *g* for molecular weight determinations in the sucrose gradient (22). Yeast alcohol dehydrogenase [mol wt 150,000 (24)] and hemoglobin [mol wt 64,500 (25)] were used as reference proteins. The position of alcohol dehydrogenase was determined by enzyme assays, and that of hemoglobin, by its absorbance at 406 nm.

RESULTS

Isolation of Cytoplasmic Aconitase from Pig Liver

As shown previously (15), separation of the more stable cytoplasmic isoenzyme from the unstable mitochondrial aconitase is readily accomplished by successive elution from DEAE-cellulose columns with 1 mM citrate (which elutes mitochondrial aconitase), followed by 5 mM citrate (which elutes cytoplasmic aconitase).

Step 1. Liver extracts containing both isoenzymes of aconitase were prepared by homogenizing 100-g lots of pig liver in 300 ml of 1 mM citrate (brought to pH 7.5 with

Tris base) and 65 ml of chloroform in a Waring Blendor for 25 sec at 0–4°. After centrifugation at 16,000 × *g* for 15 min at 0–4°, supernatant fractions were collected for column chromatography and gassed with N_2. For each column, extracts of 400 g of liver (approximately 1200 ml) were used.

Step 2: DEAE-cellulose chromatography. All further operations were performed at 4°. Approximately 1200 ml of supernatant fraction (from step 1) were placed on a DEAE-cellulose column (5 × 54 cm) that had been equilibrated at 0–4° with 1 mM citrate (brought to pH 7.5 with Tris base) and gassed with N_2. A steady stream of N_2 was maintained through the supernatant during addition to the column. Elution of the mitochondrial isoenzyme was completed by 1550 ml of 1 mM citrate (plus Tris base adjusted to pH 7.5). The cytoplasmic enzyme was eluted by 5 mM citrate (plus Tris base brought to pH 7.5) as described previously (15). The fractions containing the cytoplasmic enzyme were combined and concentrated 20-fold at 0–2° with the aid of an Amicon Diaflow filter system (PM-30 membrane).

Step 3. The cytoplasmic enzyme concentrate from step 2 was diluted 1:5 with double-distilled water at 0° and placed on a second DEAE-cellulose column (2.5 × 50 cm, equilibrated with 1 mM citrate, brought to pH 7.5 with Tris base) under an N_2 atmosphere. The cytoplasmic aconitase was eluted by a linear gradient of citrate–Tris mixture, generated by mixing of 1 liter of 5 mM Tris base and 1 mM citrate (adjusted at 25° to pH 7.5 with HCl) with 1 liter of 100 mM Tris base plus 1 mM citrate (adjusted to pH 7.5 with HCl at 25°). Combined fractions containing the cytoplasmic aconitase were concentrated by filtration, as in the preceding step.

Step 4. The enzyme concentrate from step 3 was passed through a Bio-Gel P-6 column (1.5 × 28.5 cm) equilibrated with 1 mM citrate (adjusted to pH 7.5 with Tris base) under an N_2 atmosphere. Effluents containing the enzyme were combined and rechromatographed on a DEAE-cellulose column (1.0 × 4.5 cm) equilibrated with 1 mM citrate (brought to pH 7.5 with Tris base). Aconitase was eluted from the DEAE-cellulose column with a linear gradient of citrate, generated from 100 ml of 1 mM citrate and 100 ml of 5 mM citrate (each citric acid solution was titrated to pH 7.5 with Tris base). The enzyme was concentrated by filtration as in step 3, through an Amicon PM-30 membrane. The final citrate concentration was adjusted to 1 mM (brought to pH 7.5 with Tris base). The enzyme was stored in divided lots at −78°.

As shown in Table 1, the specific activity of the final preparation increased 80–90-fold above the starting material. It should be noted that the liver extract (step 1) contained both isoenzymes, and because only about 50% of the total enzymatic activity corresponded to the cytoplasmic enzyme (15), the degree of purification of cytoplasmic aconitase was probably close to 160-fold. It also follows that the apparent recovery of 5% based on the total aconitase activity of the liver extract (step 1) should more probably be 10% for the cytoplasmic isoenzyme.

The cytoplasmic aconitase obtained in step 4 gave no increase in specific activity when subjected to further chromatography on Bio-Gel A or P-300. Its activity was not affected by 25 mM cysteine plus 1.25 mM Fe^{2+} (15). However, incubation for a few hours, even at 0°, resulted in a progressive loss of aconitase activity, which could be recovered by the addition of the Fe^{2+}-cysteine activator system. The cytoplasmic aconitase at pH 8.3 gave only one enzymatically active, electrophoretically separable protein band on acrylamide gel (Fig. 1). The enzyme could not be eluted from carboxymethyl cellulose with 100 mM citrate (adjusted to pH 5.0 with Tris base), indicating its basic character

TABLE 1

Purification of cytoplasmic aconitase from pig liver

Purification step	Total protein	Total aconitase activity at 25°	Specific activity at 25°
	mg	*μmoles/min*	*μmoles/min/mg protein*
Step 1 (liver extract)	12,300	1,645	0.134
Step 2	560	730	1.32
Step 3	180	500	2.76
Step 4	8	85	10.6

FIG. 1. *Polyacrylamide electrophoresis pattern of purified cytoplasmic aconitase (step 4, Table 1)*

The left half of the column is stained for enzymatic activity. The right half is stained for protein (see *Methods*). The apparent shadows on the right half column are photographic artifacts.

under these conditions. The enzyme gave a symmetrical peak on ultracentrifugation in a sucrose density gradient, and from its mobility as compared to hemoglobin an apparent molecular weight of 111,000 was calculated. Based on comparison with yeast alcohol dehydrogenase, this value was 107,000. This molecular weight is higher than found for aconitase of pig heart, which was 89,000 as determined by sedimentation equilibrium ultracentrifugation (5). Spectral properties of the purified cytoplasmic liver enzyme were very similar to those of the heart aconitase (5). The calculated absorbance for a 1% solution of cytoplasmic aconitase at 280 nm (1-cm light path) was 13–14. Without any activator, the turnover of the cytoplasmic liver enzyme at pH 7.5 was 19.2 sec^{-1}, calculated as *cis*-aconitate formation from citrate at 25°, with 30 mM citrate (pH adjusted to 7.5 with Tris base) as substrate. This is comparable to a turnover of 13.5 sec^{-1} of the fully activated purified pig heart enzyme (5), calculated as isocitrate formation from citrate.

Partially Purified Mitochondrial Aconitase

The restriction imposed by the avoidance of Fe^{2+}-cysteine as an activator limited the purification of the mitochondrial isoenzyme. Similar mitochondrial aconitase preparations were obtained by elution of the enzyme from DEAE-cellulose with 1 mM citrate (see step 1) or by direct extraction of isolated mitochondria with 1 mM citrate (15). The separated mitochondrial enzyme (eluted with 1550 ml of 1 mM citrate) from step 2 was concentrated 20-fold by filtration and used without further purification for enzyme kinetic studies. Storage at −78° of the concentrated mitochondrial aconitase-containing extract preserved activity for several weeks, but a subsequent slow decay of enzymatic activity could not be prevented. Activity was recovered by Fe^{2+}-cysteine (15). However, the freshly prepared mitochondrial extract required no Fe^{2+} activator when used in this crude state. Further purification attempts to increase specific activity resulted in significant loss of enzymatic activity and in a dependence on Fe^{2+}-cysteine

for full activity. For the purpose of the present work, further purification and attempts at quantitative recovery of the enzyme were abandoned. The specific activity of the mitochondrial preparation used in inhibition studies was 0.212, corresponding to about 4-fold purification.

Steady-State Analysis of Inhibition by (−)-erythro-Fluorocitrate

When initial rates of *cis*-aconitate formation (10) were determined from either citrate or isocitrate within a range of 10–5000 μM tricarboxylic acid substrates, a completely regular Michaelis-Menten type of substrate-velocity relationship was obtained. The inhibitor (−)-*erythro*-fluorocitrate changed only the apparent substrate constants (K_m), and double-reciprocal plots of initial velocities (see *Methods*) unambiguously indicated competitive inhibition with respect to both tricarboxylic acid substrates (Fig. 2). Substrate and inhibitor constants are shown in Table 2. Above 6 mM, *dl*-isocitrate exhibited apparent substrate activation with the mitochondrial isoenzyme only. Because this concentration of isocitrate was far above the expected physiological ranges of isocitrate content of cellular systems, the observed kinetic anomaly was not further investigated.

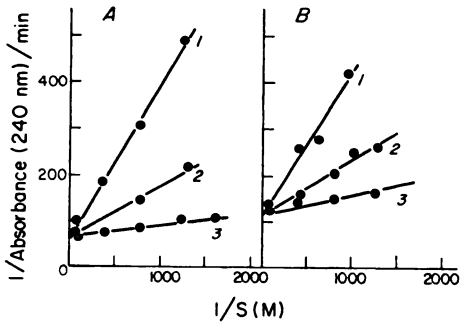

FIG. 2. *Competitive inhibition of cytoplasmic (A) and mitochondrial (B) aconitase of pig liver by (−)-erythro-fluorocitrate*

Rates of *cis*-aconitate formation from citrate were measured at 240 nm (10) in 0.15 M Tris-HCl, pH 7.5, at 25°. In A, 1.5 μg (protein) of cytoplasmic aconitase, and in B, 32 μg of mitochondrial aconitase, were used per test system (5-cm light path; 3-ml volume) at varied concentrations of citrate (abscissa). Curve *1*, 500 μM fluorocitrate; *2*, 100 μM fluorocitrate; *3*, no fluorocitrate.

TABLE 2

Substrate constants for citrate and d-isocitrate and inhibitor constants for (−)-erythro-fluorocitrate determined at pH 7.5 (10 mM Tris-HCl) and 25°

Isoenzyme	K_m			K_i		
	Citrate		*d*-Isocitrate (A)	Citrate		*d*-Isocitrate (A)
	A[a]	B		A	B	
	μM		μM	μM		μM
Cytoplasmic	220	700	17	18	27	20
Mitochondrial	420	250	17	66	57	35

[a] A, determined by the *cis*-aconitate assay; B, determined by the isocitrate dehydrogenase assay (Mg^{2+} concentration varied between 0.1 and 5.3 mM).

The K_m for citrate and K_i for (−)-*erythro*-fluorocitrate were determined again in the isocitrate dehydrogenase coupled assay (see *Methods*) in the presence of Mg^{2+} as the activator of isocitrate dehydrogenase. For the calculation of free tricarboxylate ions, the association constants of the citrate-Mg^{2+} and fluorocitrate-Mg^{2+} complexes were determined at pH 7.5 and 25° and found to be 1920 M^{-1} and 850 M^{-1}, respectively. When the concentrations of free citrate and fluorocitrate were calculated in the isocitrate dehydrogenase coupled assay at various Mg^{2+} concentrations (between 0.1 and 5.3 mM), the K_m of citrate for the cytoplasmic enzyme was 700 μM and 250 μM for the mitochondrial enzyme. These values agree within 2–3-fold with those determined with the *cis*-aconitate assay in the absence of Mg^{2+}. It is apparent, therefore, that the K_m of citrate is not modified by the presence of the Mg^{2+}-citrate complex and that the citrate anion is the true substrate of the enzyme. The K_i values for (−)-*erythro*-fluorocitrate, calculated under the same conditions, were 27 μM for the cytoplasmic aconitase and 57 μM for the mitochondrial aconitase. These values are in good agreement with K_i values determined in the absence of Mg^{2+} (Table 2). It is also evident that in the isocitrate dehydrogenase coupled system, other ligands ($NADP^+$, isocitrate dehydrogenase) do not interfere when

kinetic constants are calculated from initial velocities.

Time Course of Aconitase Activity in the Presence of Mg^{2+} or Mn^{2+}

Initially linear rates of enzymatic reactions deviate from linearity beyond 1–2 min when fluorocitrate and Mg^{2+} or Mn^{2+} are present simultaneously. This was first observed in the isocitrate dehydrogenase coupled assay system (7). That inhibition by fluorocitrate in the coupled assay system was due to aconitase inhibition was apparent from experiments which showed that fluorocitrate does not inhibit isocitrate dehydrogenase (3, 7). The effects of 10 μM fluorocitrate were determined in the coupled assay system (Fig. 3) on both aconitase isoenzymes in the presence of 0.5 and 5 mM Mg^{2+} as the activator of isocitrate dehydrogenase. Whereas linear rates were maintained for up to 10 min with 0.5 or 5 mM Mg^{2+} alone (curves *1* and *2*), enzymatic activities were progressively inhibited when 10 μM (−)-*erythro*-fluorocitrate and 0.5 or 5.0 mM Mg^{2+} were present simultaneously (Fig. 3, curves *3* and *4*). The effects of 1 and 10 μM (−)-*erythro*-fluorocitrate are shown in Fig. 4 with 5 mM Mn^{2+} as the activator of isocitrate dehydrogenase. As with Mg^{2+}, inhibition increased with time, but in the presence of Mn^{2+} apparent initial rates were also inhibited to a degree unpredictable from substrate and inhibitory constants (see Table 2). Addition of 30 mM citrate at 15 min reversed the inhibitory effect observed in the presence of (−)-*erythro*-fluorocitrate and 5 mM Mn^{2+} (Fig. 4, curves *2* and *3*). The reversal was complete at 1 μM and partial at 10 μM fluorocitrate concentration. As expected from K_m values, addition of 30 mM citrate to controls did not significantly alter the course of enzymatic reaction in the first 15–20 min (not shown).

From Figs. 3 and 4 it is clear that Mg^{2+} and especially Mn^{2+} seem to augment the inhibitory effect of fluorocitrate on aconitase in the isocitrate dehydrogenase coupled assay system. It was of importance to determine the effect of Mn^{2+} on the activity of aconitase in an assay system not dependent on Me^{2+}. The effect of Mn^{2+} on the rate of *cis*-aconitate formation from citrate was measured spectrophotometrically at 240 nm (10). This is shown in Fig. 5, where curves *1* and *2* are rates obtained with the cytoplasmic and mitochondrial isoenzymes, respectively, in

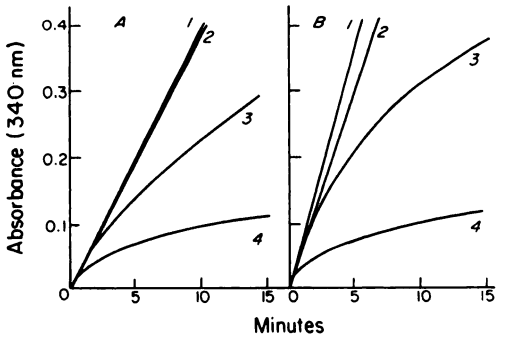

FIG. 3. *Time-dependent inhibition of aconitase cytoplasmic (A) and mitochondrial (B) isozymes by 10 μM (—)-erythro-fluorocitrate in the presence of 0.5 mM Mg^{2+} (curves) 3) and 5 mM Mg^{2+} (curves 4).*

Controls (curves *1* and *2*) contained no fluorocitrate; only 0.5 and 5 mM Mg^{2+}, respectively. Aconitase activity was measured in the isocitrate dehydrogenase coupled assay (see *Methods*). The substrate was 1 mM citrate. Notice that at this fluorocitrate to citrate ratio no inhibition of initial velocity is expected. Conditions are described in the legend to Fig. 2, except that spectrophotometric readings were recorded at 340 nm. Figures 3–5 are reproductions of actual chart recordings.

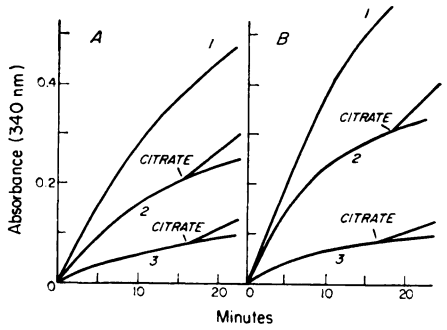

FIG. 4. *Inhibitory effects of 1 μM (curves 2) and 10 μM (−)-erythro-fluorocitrate (curves 3) on cytoplasmic (A) and mitochondrial (B) aconitase isoenzymes in the presence of 5 mM Mn^{2+}*

At 15 min, 30 mM citrate was added. Curves *1* are the controls containing 5 mM Mn^{2+} but no fluorocitrate. Conditions were the same as described in the legend to Fig. 3. As in earlier studies (7), each test system contained 1 mM reduced glutathione.

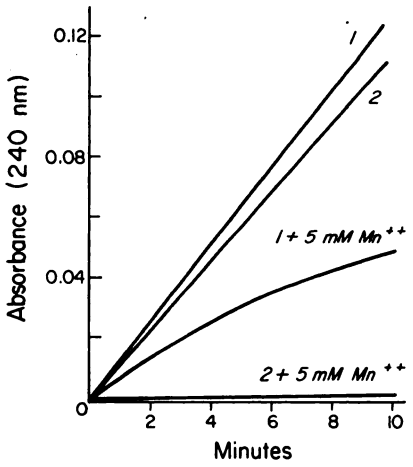

FIG. 5. *Effect of 5 mM Mn^{2+} on rate of cis-aconitate formation from 1 mM citrate, catalyzed by cytoplasmic (curve 1) and mitochondrial (curve 2) aconitase isoenzymes*

Experimental conditions are described in the legend to Fig. 2.

the absence of Mn^{2+}. The presence of 5 mM Mn^{2+} diminished the activity of the cytoplasmic enzyme by about 50% [curve *1*(+5 mM Mn^{2+}), Fig. 5] and completely inhibited the mitochondrial isoenzyme [curve *2* (+5 mM Mn^{2+}), Fig. 5].

DISCUSSION

Steady-state analyses carried out with both aconitase isozymes of pig liver yielded K_i values for (−)-*erythro*-fluorocitrate about 6–12 times lower than reported by Villafranca and Mildvan (5) and Glusker (6) for the purified aconitase isolated from pig heart. It is of importance that our K_m and K_i values when calculated from association constants of citrate and fluorocitrate-Mg^{2+} complexes were identical with those obtained in the absence of added Me^{2+}. Despite the large differences in the purity and stability of mitochondrial and cytosol isozymes, nearly the same results were obtained with both enzymes, provided that initial velocity conditions were carefully observed (see *Methods*). It is therefore suggested that the very large differences between the K_i values reported by Brand *et al.* (9) and found by others (5–7) are most probably due to the techniques used for determination of initial velocities. Although this point cannot be elucidated from the paper of Brand *et al.* (9), it is assumed that the aconitase reaction might have been initiated by the addition of citrate to disrupted mitochondrial systems exposed to Mg^{2+} as a necessary component of the isocitrate dehydrogenase coupled assay employed by these workers. As expected, inhibition of aconitase by Mg^{2+} would have taken place under these conditions, and the reinforced inhibition by Mg^{2+} plus fluorocitrate (see Fig. 3) would have necessarily resulted in nonlinear reaction rates, unsuitable for the determination of K_i values, which have to be based on linear initial velocities. Double-reciprocal plots of anomalous rates may easily result in large errors in the computation of K_i when velocity and substrate constants are substituted into rate equations derived on the basis of initial rate measurements of enzymatic reactions which follow a Michaelis-Menten mechanism. An additional error is introduced when, instead of free citrate (calculated from the association constant with Mg^{2+}), total added citrate is plotted against nonlinear rates.

Our results as well as others' (5–7) indicate that (−)-*erythro*-fluorocitrate is not an impressive inhibitor of aconitase (K_i is near the K_m of isocitrate), and this experimental fact tends to diminish the probability that this reversible, linearly competitive inhibition is the most sensitive cellular target site of fluorocitrate. In contrast to the instability of extracted mitochondrial aconitase, intact mitochondria sustain their ability to catalyze the Krebs pathway of citrate metabolism for prolonged periods and maintain stable aconitase as determined by citrate-isocitrate flux (26); therefore it is unlikely that instability of extracted aconitase *in vitro* can be extrapolated to intact mitochondria. The Mg^{2+} content of the matrix space of liver mitochondria is 10–18 nmoles of Mg^{2+} per milligram of protein,[3] and it is highly probable that almost all Mg^{2+} is present in form of nucleotide or other complexes. It is therefore unlikely that Mg^{2+} or Mn^{2+}, as present in mitochondria, can contribute to aconitase instability, which in fact is not found with these particles.

[3] E. Kun, unpublished observations.

As observed by us (26–28), the large inhibition of citrate-isocitrate flux which occurs when intact mitochondria are first incubated with micromolar or lower concentrations of (−)-*erythro*-fluorocitrate is abolished when the same mitochondria are dissolved in Triton X-100. Similar results were reported by Brand *et al.* (9). If this inhibition were explainable by inhibition of mitochondrial aconitase, then disruption of mitochondrial membranes (by the detergent) should not have removed the inhibition by fluorocitrate when the apparent aconitase activity of *intact* mitochondria and enzyme activity of the same mitochondrial *solution* were measured successively in the same test system. The only explanation left is the assumption that intact mitochondria can concentrate added fluorocitrate over a range of several orders of magnitude to approach intramitochondrial inhibitor concentrations corresponding to K_i values obtained with the isolated enzyme. Although this possibility cannot be strictly ruled out, several observations argue against the assumption that the ensuing inhibition corresponds to the competitive mechanism as determined with isolated aconitase. First, citrate, if present prior to fluorocitrate, prevents the inhibition of citrate-isocitrate flux of intact mitochondria (26). Whereas this phenomenon explains the observation of Brand *et al.* (9), who found that fluorocitrate did not inhibit citrate exchange in citrate-loaded mitochondria, it does not agree with a competitive inhibitory mechanism of aconitase, in which the degree of inhibition depends only on the substrate-to-inhibitor ratio and not on the order of their addition. Second, the inhibition of citrate-isocitrate flux of intact mitochondria by incubation with low fluorocitrate concentrations, which do not correspond to the K_i of fluorocitrate with respect to aconitase, is apparently irreversible (26, 27) (i.e., cannot be reversed by further addition of even high concentrations of citrate). This phenomenon further argues against the assumption that competitive inhibition of aconitase alone explains the mode of action of fluorocitrate on mitochondria, and suggests that other systems, such as membrane-associated translocation, are also involved. The discrepancy between the K_i of fluorocitrate for isolated aconitase and the much greater sensitivity of intact mitochondria to fluorocitrate, as well as the apparently irreversible effect of fluorocitrate on mitochondria, constitutes the experimental basis of further investigations. Determination of the site of action of fluorocitrate on mitochondrial systems will necessarily depend on the isolation of components of the substrate carrier apparatus and elucidation of its coupling to energy-linked membrane functions, a work presently pursued.

Our preliminary experiments, related to the mode of action of Me^{2+} [4] on aconitases, permit only tentative conclusions. High concentrations of citrate can prevent and reverse the inhibition by Me^{2+}. Because the concentration of citrate (30 mM) required to reverse the inhibition by Mn^{2+} bears no relationship to the K_m of citrate, it seems probable that both Me^{2+} and high concentrations of citrate act on some modifier site(s) of isolated aconitase. We presume that fluorocitrate competes with citrate for the modifier site(s) and diminishes the enzyme-stabilizing effect of citrate against inactivation by Me^{2+}. Two experimental facts support this view: citrate is a necessary enzyme stabilizer required during isolation, and fluorocitrate has a lower affinity toward Mg^{2+} than citrate; thus it would be expected to be a less potent antagonist than citrate to inactivation by Me^{2+}.

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[4] Me^{2+} refers to Mg^{2+} or Mn^{2+}.

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