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α-Methylisocitrate

A SELECTIVE INHIBITOR OF TPN-LINKED ISOCITRATE DEHYDROGENASE FROM BOVINE HEART AND RAT LIVER*

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GERHARD W. E. PLAUT, RICHARD L. BEACH,‡ AND TADASHI AOGAICHI

From the Department of Biochemistry, Temple University School of Medicine, Philadelphia, Pennsylvania 19140, and the Chemistry Department, Rider College, Trenton, New Jersey 08602

α-Methylisocitrate (3-hydroxy-1,2,3-butanetricarboxylate) is a potent inhibitor, competitive with isocitrate (1-hydroxy-1,2,3-propanetricarboxylate), of the TPN-linked isocitrate dehydrogenase from bovine heart and rat liver; it does not inhibit the DPN-specific enzyme from these tissues. In the presence of magnesium ion, values of K_{is} for DL-α-methylisocitrate for purified bovine heart enzyme, rat liver cytosol, and rat liver mitochondrial extract were in the range of 0.1 μM to 0.3 μM. This compared to values of apparent K_m for DL-isocitrate for the same tissue preparations of 14 μM to 20 μM. One of the DL isomer pairs of α-methylisocitrate was inactive; the observations suggest that it is *threo*-α-methylisocitrate which inhibits TPN-linked isocitrate dehydrogenase. A method of synthesis of DL-*threo*-α-methylisocitric lactone (2-methyl-5-oxo-2,3-furandicarboxylic acid) from dimethyl *trans*-epoxymethylsuccinate and dimethylmalonate is described.

In a study of the substrate activities of α-hydroxy-α,β-dicarboxylic acid derivatives for isocitrate dehydrogenases (1) attention was focused on the effect of substituents at the β carbon of these derivatives. It was found that addition of a methylene group to isocitrate, resulting in the higher homologue homoisocitrate, led to retention of substrate activity for the DPN-specific isocitrate dehydrogenase, but not the TPN-linked enzyme from bovine heart. D-Garcinia acid (2*R*,3*R*-2-hydroxycitric acid), in which the β-hydrogen of D-*threo*-isocitrate is substituted by a hydroxyl group was a substrate for both isocitrate dehydrogenases.

Substitution of the α-hydrogen of isocitrate would abolish the substrate activity of such analogues but might not prevent binding to the enzyme. Such analogues might be useful for elucidating the mechanism of action and the metabolic role of the isocitrate dehydrogenases. Formation of α-methylisocitrate and methylcitrate from α-methyl-*cis*-aconitate by the action of aconitase had been reported by Gawron and Mahajan (2). α-Methylisocitrate prepared in this manner was cleaved to pyruvate and succinate by isocitrate lyase from several organisms (3). The differential inhibition of the DPN-linked and TPN-specific isocitrate dehydrogenases from heart and liver by chemically synthesized preparations of α-methylisocitrate is the subject of the present report.

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EXPERIMENTAL PROCEDURE

Materials

DPN-linked isocitrate dehydrogenase was purified to homogeneity from lyophilized bovine heart mitochondria (4). TPN-linked isocitrate dehydrogenase from the extramitochondrial fraction of bovine heart homogenate was purified by a modification of the method of Rose (5). The final preparation had a specific activity of 8 μmol of TPNH-formed min^{-1} mg^{-1} protein at 25° under the conditions of assay described by Siebert *et al.* (6). Rat liver mitochondria were prepared by the method of Schneider and Hogeboom (7), but in a medium containing 225 mM mannitol, 75 mM sucrose, and 0.1 mM EDTA. The cytosol fraction of the liver homogenate was clarified by centrifugation at 38,000 × *g* for 60 min before assay of TPN-specific isocitrate dehydrogenase activity. Extracts of rat liver mitochondria were prepared by sonication at 5–8° in a medium containing 225 mM mannitol, 75 mM sucrose, and 0.05 mM EDTA at pH 7.0, followed by centrifugation at 150,000 × *g* for 30 min to remove submitochondrial particles.

Assays

DPN-linked isocitrate dehydrogenase activity was determined spectrophotometrically at 25° as described previously (4), in a medium containing 166 mM Na-Hepes[1] at pH 7.4, 0.3 mM ADP, and 0.33 mM DPN^+; the concentrations of DL-*threo*-isocitrate, divalent metal ion activators, and α-methylisocitrate were as reported in the text and table. TPN-linked isocitrate dehydrogenase in soluble enzyme preparations was determined at 25°, as described by Siebert *et al.* (6) in a medium containing 167 mM Na-Hepes at pH 7.4 and 0.10 mM TPN^+; the concentrations of DL-*threo*-isocitrate, divalent metal ions, and α-methylisocitrate are reported in the legends of tables and figures. TPN-linked isocitrate dehydrogenase of intact liver mitochondria was determined under the conditions of Hogeboom and Schneider (8).

[1] The abbreviation used is: Hepes, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid.

Kinetic parameters for the soluble enzyme preparations were obtained by fitting the experimental initial velocities to the appropriate equation using the computer programs developed by Cleland (9).

α-Methylisocitric lactone and isocitric lactone were hydrolyzed under alkaline conditions before assay, as described previously (10).

Preparation of α-Methylisocitrate

DL-α-Methylisocitric lactone (2-methyl-5-oxo-2,3-furandicarboxylic acid) was prepared from dimethyl *trans*-epoxymethylsuccinate and dimethylmalonate by an adaptation of the procedure of Gawron *et al.* (11) for the preparation of isocitric lactone. This synthesis, described below, should favor the formation of the DL-*threo* compound, and the product showed a single component by electrophoresis on Whatman No. 3MM paper (or ChromAR) in 0.06 M pyridine 0.79 M acetate buffer at pH 3.5 (1.5 hours at 1900 volts). α-Methylisocitric lactone synthesized by the procedure of Rach (12) exhibited two components by electrophoresis and could be separated into two fractions (Fraction A and Fraction B) by fractional crystallization from ethyl acetate. The elementary analysis and neutralization equivalents of either fraction corresponded to the composition expected for α-methylisocitric lactone. However, the single component found by paper electrophoresis of Fraction B corresponded in migration to the compound synthesized by the method described below, whereas Fraction A migrated more rapidly toward the anode in buffers between pH 3 and pH 4. Furthermore, the NMR spectra of the newly synthesized compound and Fraction B were identical, but differed from Fraction A.

2-Methyl-5-oxo-2,3-furandicarboxylic Acid (α-Methylisocitric-γ-lactone)

Dimethyl trans-epoxymethylsuccinate—Diazomethane generated from Diazald is distilled into a 500-ml Erlenmeyer flask containing 7.21 *g* of *trans*-epoxymethylsuccinic acid (49 mmol) (13) suspended in 20 ml of anhydrous diethyl ether. The reaction mixture is cooled in an ice bath and stirred with a magnetic stirrer throughout the addition. Diazomethane addition is stopped when the reaction reaches completion, as indicated by the yellow color of diazomethane in the reaction flask. The excess diazomethane is destroyed by the addition of 2 to 3 drops of glacial acetic acid, and the ether is removed by distillation, yielding 8.85 g of a clear syrup. The product (5.6 g, 67%) may be distilled at 80° 0.9 torr.

$C_7H_{10}O_5$

Calculated: C 48.28, H 5.79
Found C 48.26, H 5.69

2-Methyl-5-oxo-2,3,4-tricarbomethoxytetrahydrofuran—To a stirred solution of sodium methoxide at 5° (prepared from 0.7 g of sodium metal (0.03 g atom) and 15 ml of dry methanol) are added 4.01 g (30.4 mmol) of redistilled dimethylmalonate. After a white precipitate forms, 5.3 g (30.4 mmol) of dimethyl *trans*-epoxymethylsuccinate are added, and the mixture is stirred for 3 days at room temperature.

Concentrated HCl (75 ml) is then added to the reaction mixture, and stirring is continued for an additional 2 hours. The NaCl precipitate is removed by filtration, and the reaction mixture is concentrated to a syrup at 45° under reduced pressure. The product (4.3 g, 51.5%) distills at 160–170° at 1.7 torr.

$C_{11}H_{14}O_8$

Calculated: C 48.18, H 5.15
Found: C 48.15, H 4.67

2-Methyl-5-oxo-tetrahydrofuran-2,3-dicarboxylic acid—One gram (3.65 mmol) of 2-methyl-5-oxo-2,3,4-tricarbomethoxytetrahydrofuran is refluxed in 40 ml of 6 N HCl for 3 hours. The solvent is evaporated in a vacuum at 50°, and residual HCl is removed completely from the product by repeated vacuum distillations following addition of 10-ml portions of water to the residue. The solid is sublimed at 180° under reduced pressure (1.5 torr). A solution of the solid in acetone is treated with Norit A, filtered, and concentrated to dryness. The residue is heated for 1 hour at 100° under reduced pressure (1 torr). The substance is crystallized from a mixture of 0.7 ml of acetone and 7 ml of benzene. The crystalline compound was recovered in a yield of 242 mg (35%).

The following analytical data were obtained for the α-methylisocitric lactone preparations.

$C_7H_8O_6$

Calculated: C 44.69, H 4.29

(Equivalent weight calculated cold: 94.1 calculated heated excess base, 62.7).

Present preparation

Found: C 44.40, H 4.32

Melting point 183–185° decomposition; equivalent weight cold, 95.9; equivalent weight heated excess base, 65.5. NMR (acetone d_6) ppm 1.88 (singlet, 3H), 2.88–3.19 (multiplet, 2H), 3.50–3.97 (multiplet, 1H).

Rach preparation

Found: C 44.67, H 4.32

Melting point, 168°–171° (decomposition), literature 168°. Equivalent weight (cold) 94.1; equivalent weight (heated) 67.9.

Fraction A

Found: C 44.97, H 4.38

Melting point 196–202° (decomposition); equivalent weight (cold) 94.5; equivalent weight (heated) 64.7. NMR (acetone d_6) ppm 1.70 (singlet, 3H), 2.93–3.07 (doublet, 2H, J = 6 cps), 3.77–3.99 (triplet, 1H J = 6 cps).

Fraction B: melting point 183–186° (decomposition) equivalent weight (cold) 96.6; equivalent weight (heated) 64.1. NMR identical to preparation synthesized by new procedure.

Unless specified otherwise, DL-α-methylisocitrate prepared by the new method was used in these studies.

RESULTS AND DISCUSSIONS

TPN-linked Isocitrate Dehydrogenase—α-Methylisocitrate was an effective inhibitor for the dehydrogenase from rat liver cytosol and for enzyme purified from the extramitochondrial fraction of bovine heart. The inhibition was competitive with respect to isocitrate (Fig. 1). When activated by magnesium ion, apparent K_m for DL-isocitrate ranged from 13.9 μM to 20 μM and K_{is} for DL-α-methylisocitrate was between 0.23 μM and 0.30 μM for the heart enzyme; the corresponding values for liver cytosol were 14.7 μM to 18.5 μM for K_m and about 0.10 μM for K_{is} (Table I).

α-Methylisocitrate also inhibited the TPN-specific isocitrate dehydrogenase activity from rat liver mitochondria, and under identical assay conditions, the values of apparent K_m for isocitrate and of K_{is} for α-methylisocitrate were nearly identical for enzyme activities of extracts of sonicated mitochondria and of the extramitochondrial fraction from rat liver homogenate. In the presence of 4.0 mM magnesium ion, the values were 15.9 μM and 14.7 μM for K_m and 0.10 μM and 0.10 μM for K_i with the mitochondrial extract and the supernatant fraction, respectively (Table I). Inhibition by α-methylisocitrate of the TPN-linked isocitrate dehydrogenase activity of intact rat liver mitochondria was observed when assayed by the procedure of Hogeboom and Schneider (8). However, measurements with the suspensions were not precise enough for kinetic comparison with activities of the rat liver extract preparations.

α-Methylisocitrate is a very potent inhibitor of TPN-linked isocitrate dehydrogenase; depending on incubating conditions, K_m/K_{is} ratios between 10 and 185 were observed (Table I). The values of apparent K_m for isocitrate were lower with Mn^{2+} than Mg^{2+}, in accord with previous results that Mn^{2+} is a more effective activator of the heart enzyme than Mg^{2+} (14); however, K_{is} for α-methylisocitrate was lower when Mg^{2+} was used instead of Mn^{2+} for activation (Table I). A similar, although less pronounced, effect of the divalent cations on inhibition constants was observed with the enzyme activity

from liver cytosol. The chelate of isocitrate with divalent metal ion activators has been reported to be the actual substrate for TPN-linked isocitrate dehydrogenase from porcine heart (15). It is possible that the chelates of α-methylisocitrate are likewise the actual inhibitors of the enzyme. The stability constants of divalent metal complexes of α-methylisocitrate are unknown; however, the large excess of divalent cations over α-methylisocitrate and isocitrate in the assay media would favor most of the α-methylisocitrate being in the metal complex form under the incubation conditions used (Table I).

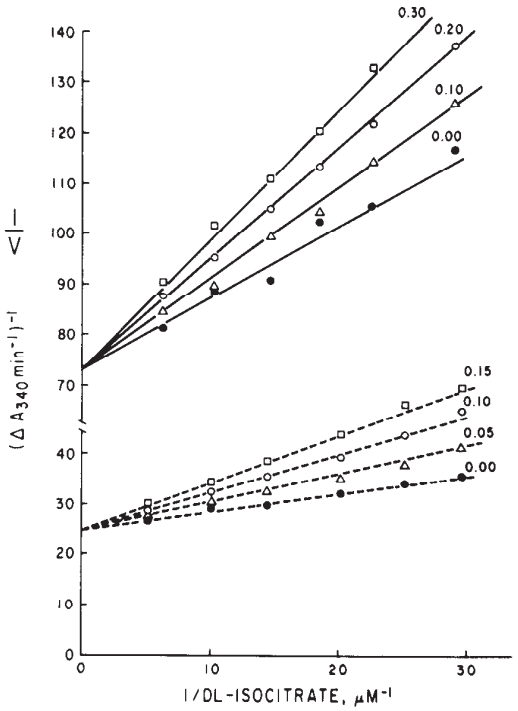


FIG. 1. Inhibition of TPN-linked isocitrate dehydrogenase. Conditions of incubation are described under "Experimental Procedure." Data are shown as double reciprocal plots of velocity *versus* DL-isocitrate, and the fixed concentrations of DL-α-methylisocitrate (μM) are shown above the lines. ——, purified enzyme from bovine heart incubated in the presence of 6.67 mM magnesium ion. Calculated values of apparent K_m for DL-isocitrate and K_{is} for DL-α-methylisocitrate were 20.0 ± 2.0 μM and 0.30 ± 0.05 μM, respectively. – – –, supernatant fraction from rat liver cytosol incubated in the presence of 4.0 mM magnesium ion. Calculated values of apparent K_m for DL-isocitrate and K_{is} for DL-α-methylisocitrate were 14.7 ± 1.47 μM and 0.096 ± 0.006 μM, respectively.

DPN-linked Isocitrate Dehydrogenase—In contrast to the marked sensitivity of the TPN-specific enzyme to α-methylisocitrate, the DPN-linked enzyme was essentially unaffected. It is difficult to demonstrate complete inactivity of the analogue, since it competes with isocitrate for activating divalent cations. Kinetic studies have suggested that magnesium isocitrate$^-$ is the actual substrate for the DPN-specific enzyme from bovine heart, and that free Mg^{2+} causes inhibition which is competitive with respect to magnesium isocitrate (16). At the relatively high concentrations of α-methylisocitrate necessary to demonstrate inhibition, decrease of activity could be due to depletion of the substrate isocitrate complex. In the experiments shown (Table II), the ratios of concentrations of total isocitrate to total divalent metal ions were adjusted to maintain relatively low constant concentrations of inhibiting free divalent cations as the concentrations of substrate and α-methylisocitrate varied. Under these conditions, α-methylisocitrate appeared inert as an inhibitor of the enzyme from rat liver mitochondria and from bovine heart with either Mg or Mn as activator.

Activity of Isomers of α-Methylisocitrate—Preparation of α-methylisocitrate by the cyanohydrin synthesis of Rach (12) should yield DL-*threo*-α-methylisocitrate and DL-erythro-α-methylisocitrate (Scheme I) in about equal amounts. α-Methylisocitrate synthesized by the method of Rach was about one-half as active as an inhibitor of TPN-linked isocitrate dehydrogenase as the newly synthesized compound (Table III).

α-Methylisocitric lactone synthesized by the Rach procedure was separated into Fraction A and Fraction B by fractional crystallization. Fraction A did not inhibit TPN-specific isocitrate dehydrogenase, whereas Fraction B and the present preparation showed equal inhibition at equivalent concentrations (Table III). The following observations suggest that *threo*-α-methylisocitrate is the active inhibitor. (*a*) The new method of synthesis should favor formation of the *threo* isomer. (*b*) In paper electrophoresis at pH 3 to pH 4, the rate of migration of Fraction A toward the anode was identical with that of DL-*erythro*-isocitric lactone, but more rapid than the migration of Fraction B (or the newly synthesized compound)

TABLE I

Inhibition of TPN-linked isocitrate dehydrogenase preparations from heart and liver

The assay conditions are described under "Experimental Procedure."

Eznyme preparation	Activating divalent cation		DL-Isocitrate	DL-α-Methylisocitrate	K_m/K_{is}
	M^{2+}	Conc.	K_m(app.)	K_{is}	
		mM	μM	μM	ratio
Purified bovine heart enzyme	Mg[a]	1.33	13.9 ± 2.1	0.23 ± 0.03	60
Purified bovine heart enzyme	Mg[a]	6.67	20.0 ± 2.0	0.30 ± 0.05	67
Purified bovine heart enzyme	Mn[b]	0.27	6.1 ± 1.9	0.60 ± 0.18	10
Purified bovine heart enzyme	Mn[b]	1.33	5.7 ± 1.2	0.52 ± 0.11	11
Rat liver cytosol (supernatant)	Mg[c]	1.33	18.5 ± 1.7	0.10 ± 0.01	185
Rat liver cytosol (supernatant)	Mg[c]	4.0	14.7 ± 1.5	0.10 ± 0.01	147
Rat liver cytosol (supernatant)	Mg[c]	6.67	15.5 ± 2.0	0.11 ± 0.01	141
Rat liver cytosol (supernatant)	Mn[c]	1.3	10.1 ± 1.1	0.23 ± 0.03	43
Rat liver mitochondrial extract	Mg[c]	4.0	15.9 ± 1.8	0.10 ± 0.02	159

[a] The concentrations of DL-isocitrate and DL-α-methylisocitrate were from 20 to 165 μM and from 0.1 to 0.4 μM, respectively.
[b] The concentrations of DL-isocitrate and DL-α-methylisocitrate were from 18 to 105 μM and from 0.9 to 3.5 μM, respectively.
[c] The concentrations of DL-isocitrate and DL-α-methylisocitrate were from 16 to 72 μM and from 0.05 to 0.30 μM, respectively.

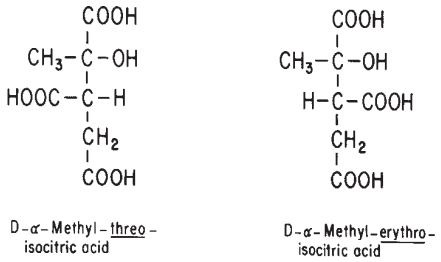
TABLE II

Effect of DL-α-methylisocitrate on DPN-linked isocitrate dehydrogenase from heart and liver

The general conditions of assay are described under "Experimental Procedure." The concentrations of free divalent cations were maintained by adjusting the concentrations of total divalent cations as described previously (16). For purposes of these approximations, it was assumed that the stability constants of the divalent metal chelates of isocitrate and α-methylisocitrate were equal.

Enzyme	Free divalent cation	DL-α-methyl-isocitrate	DL-iso-citrate	Activity[a]
	mM	*mM*	*mM*	(% ± *S.D.*)
Purified from bovine heart	Mg^{2+} (0.04–0.05)	1.5	2.0	99.8 ± 3.7 (9)
Purified from bovine heart	Mn^{2+} (0.04–0.06)	1.5	0.9	102.1 ± 9.5 (12)
Rat liver mitochondrial extract	Mg^{2+} (0.04–0.05)	1.5	2.9	88.7 ± 8.3 (4)

[a] Velocities measured at each concentration of isocitrate and α-methylisocitrate indicated were compared to the reaction rates in the absence of α-methylisocitrate. The numbers in parentheses refer to numbers of experiments. Additional experiments have been done at other levels of substrate and inhibitor; only the results obtained at the lowest levels of isocitrate (K_m(app.) total DL-isocitrate = 10 mM) and the highest concentrations of α-methylisocitrate used are reported here.



SCHEME 1

which corresponded to that of DL-*threo*-isocitric lactone. The relative rates of migration in electrophoresis of the *erythro* and *threo*- isomers would be in agreement with the finding of Gawron and Glaid (17) that the values of $pK'a_1$ and $pK'a_2$ of DL-*erythro*-isocitric lactone were lower than those of DL-*threo*-isocitric lactone. (*c*) In preliminary experiments with aconitase from bovine heart, Fraction A was inert, whereas Fraction B and the newly synthesized compound had substrate activity. Gawron and Mahajan (2) had shown that α-methyl-*cis*-aconitate was a substrate for aconitase and, on the basis of the stereospecificity of the enzyme, suggested that the isocitrate formed had the D-*threo* configuration. Presumably, the same isomer of *threo*-α-methylisocitrate in the synthetic preparations was active for aconitase. Although a resolution of the D and L isomers has not been accomplished, the substrate specificity of isocitrate dehydrogenase (18) makes it likely that D-*threo*-α-methylisocitrate is the inhibitor of the TPN-specific enzyme.

Ando *et al.* (19) have observed urinary excretion of methylcitric acid in patients with propionic aciduria and methylmalonic aciduria. They have suggested that methylcitrate or a metabolite of this compound may lead to increased ketone body formation in such patients by blocking the citric acid cycle. If the isomer of methylcitrate formed in such patients were convertible to *threo*-α-methylisocitrate by the action of aconitase, the inhibition of TPN-linked isocitrate dehydrogenase by α-methylisocitrate could contribute to increased diversion of acetyl-CoA from the citric acid cycle to ketone body formation.

TABLE III

Inhibition of TPN-linked isocitrate dehydrogenase by various preparations of DL-α-methylisocitrate

Assays were done as described under "Experimental Procedure" with purified enzyme from bovine heart in the presence of 1.33 mM $MnSO_4$, 70 μM DL-isocitrate, and α-methylisocitrate at the concentrations indicated. The samples of α-methylisocitrate lactone were hydrolyzed before use, as described under "Experimental Procedure."

Method of preparation of α-methylisocitrate	α-Methylisocitrate	Inhibition
	μM	%
New synthesis	1.75	20
New synthesis	3.5	35
New synthesis	7.0	52
Rach	1.75	8
Rach	3.50	14
Rach	7.0	25
Fraction A	7.0	0
Fraction B	7.0	54

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