

Citrate Conformation and Chelation: Enzymatic Implications

JENNY P. GLUSKER

The Institute for Cancer Research, The Fox Chase Cancer Center, Philadelphia, Pennsylvania 19111

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Citric acid was first isolated in a pure form from lemon juice by Scheele in 1784 and has been found in most plant and animal tissues, often in high concentrations. It accounts for 5% by weight of lemon juice and about 0.3% by weight of teeth and bone. Citric acid is, however, generally prepared commercially by fermentation of a mold that can convert glucose almost quantitatively to citrate. On purification, colorless crystals are formed which effloresce in dry air; when crystals are grown from warm solution, a more stable anhydrous form results. This tribasic nontoxic acid and its salts are used in a large variety of applications in everyday life: in soft drinks and effervescent salts, as antioxidants in foods, as a sequestering agent for metal binding, as a cleaning and polishing agent for metals, and as a mordant in dyeing. Many of these applications utilize the good chelating ability of citrate for cations such as calcium, strontium, manganese, magnesium, and iron.

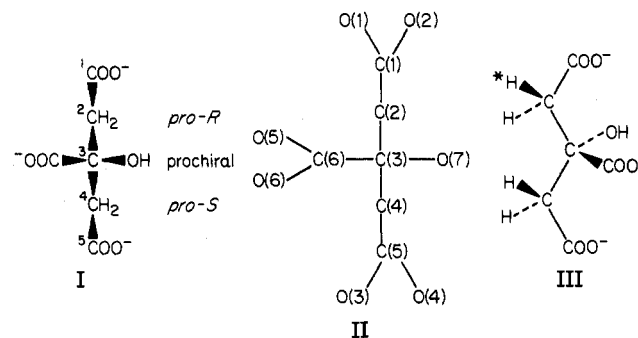
This ubiquitous acid and its salts have key physiological functions.¹ The citrate ion is an intermediate in the later stages of carbohydrate metabolism and is found in the cycle that is called the "citric acid cycle" or the "Krebs cycle".² In this cycle, the oxidation of citrate in the mitochondria leads to the production of energy. Oxaloacetate is produced and interacts with acetyl-CoA to regenerate citrate. Other metabolic functions of citrate^{3,4} include its ability to supply acetyl groups for biosynthetic pathways (such as when citryl-CoA is cleaved by cytosolic ATP citrate lyase), its use as a hydrogen donor for cytosolic syntheses, and its controlling action (activating or inhibiting) on several enzymes, including acetyl-CoA carboxylase (an enzyme needed for lipid synthesis) and phosphofructokinase (an enzyme whose reaction is the rate-limiting step in glycolysis). In addition, the chelation of calcium by citrate to give an insoluble salt (found in bone) has been considered to be of importance in calcium metabolism.

The citrate ion has posed several interesting stereochemical problems for biochemists through the years, and therefore has been an ideal candidate for three-dimensional structural studies. A description of such studies, by X-ray techniques, together with a discussion of the way in which they can be used to gain an understanding of the mechanisms of action of enzymes that act on citrate, forms the subject of this Account.

Jenny (Pickworth) Glusker was born in Birmingham, England, and received a D.Phil. degree from Oxford University in 1957. She did undergraduate research in fine structure of infrared spectra with H. W. Thompson and graduate work on the structure, by X-ray crystallographic techniques, of the hexacarboxylic acid derived from vitamin B₁₂ with D. C. Hodgkin. After postdoctoral studies on peptide structures with R. B. Corey at Caltech, she came to the Institute for Cancer Research in Fox Chase, Philadelphia, to work with A. L. Patterson. She is now a Senior Member at the Institute for Cancer Research and her research interests are enzyme mechanisms and their stereochemistry and the interactions of polycyclic carcinogens, mutagens, and antitumor agents with biological macromolecules. She was President of the American Crystallographic Association in 1979, and received the Philadelphia Section Award of the ACS in 1978 and the Garvan Medal in 1979.

General Features of the Citrate Ion

The formula of the citrate ion is shown in I, the numbering used here in II, and a general perspective in III. There are no asymmetric carbon atoms in citric



acid or its anion, but if they are viewed as in I, the carboxymethyl group ($-\text{CH}_2\text{COO}^-$) which lies toward the top of the page can always be distinguished from the lower carboxymethyl group by its relationship to the central hydroxyl and carboxyl groups; these two carboxymethyl groups are designated *pro-R* and *pro-S*, respectively, in the *R/S* system.⁵ However, the prochiral anion or acid can be made asymmetric by substitution of one of the hydrogen atoms in the methylene groups by another atom or group. The resulting analogue, such as hydroxycitrate, fluorocitrate, or methylcitrate, contains two asymmetric carbon atoms, and hence four isomers can exist. In biochemical systems, the "parent-numbering" system⁶ (which relates the analogue to its "parent" compound) is useful, because certain substitutions, as, for example, of hydrogen by fluorine, may alter the *R/S* designation of a carbon atom.

The three-dimensional structures of citric acid^{7,8} and its monohydrate^{9,10} and of several salts¹¹⁻²⁵ have been

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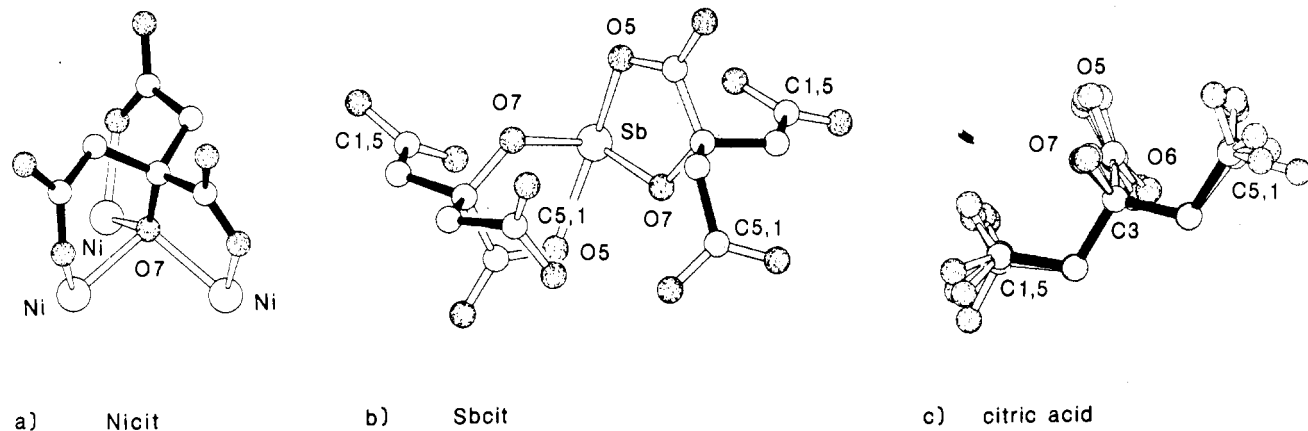


Figure 1. (a) Chelation in a complex nickel citrate illustrating an ionized hydroxyl group with three nickel cations around it. All bonds in the citrate ion are black. Nickel-oxygen contacts are open bonds. Note the tetrahedral coordination of the hydroxyl group, O(7). (b) Chelation in an antimony citrate (Sbcit(I) and Sbcit(II)). The backbone from C(1) to C(5) is black. Note the two different backbone conformations, extended (left-hand side) or with one end turned in (right-hand side). (c) Citric acid and its monohydrate and their mirror images superimposed on each other. The backbone is black. Note that the variation in conformation is slight for the central α -hydroxycarboxylic acid group and great for the terminal carboxyl groups.

studied by X-ray diffraction methods. There are four ionizable groups in citric acid (three carboxyl groups, $pK_1 = 3.13$, $pK_2 = 4.76$, $pK_3 = 6.40$,²⁶ and a hydroxyl group with a value of 11 or greater for pK_4).²⁷ In the *solid* state, it is found that the central carboxyl group (α to a hydroxyl group) is ionized first, then the two terminal carboxyl groups, and finally the hydroxyl group. NMR studies²⁸ indicate that this order of ionization is generally found in solution as well. Although the pK_a for the ionization of the hydroxyl group is high, the existence of an ionized hydroxyl group is clearly seen in two of the three anions in a nickel citrate complex.²⁴ In these the hydroxyl group is surrounded tetrahedrally by three different nickel cations at approximately equal distances, as shown in Figure 1a. This completes tetrahedral coordination around O(7), leaving no possible place for a hydroxyl hydrogen atom.

There are two conformations of citrates that are to be found among the structures determined to date, as shown by the examples in Figure 1b,c and tabulated in the list of torsion angles in Table I. The backbone of citrate, from C(1) to C(5), may be fully extended (T1 and T2 both near 180°) in a planar zigzag or else one terminal carbon atom (C(1) or C(5)) may be swung around (T1 or T2 near $\pm 60^\circ$).

The C(3)–C(6) bond is typically longer than the value

of 1.511 Å normally found for a C(sp³)–C(sp²) bond. The average value for the C(3)–C(6) bond in citrates is 1.544 Å, 8 estimated standard deviations larger than the expected value. This lengthening of a carbon-carbon bond is reminiscent of the "polymethine state" described by Kulpe²⁹ for sp² carbon atoms. It implies the same charge (presumably a slight positive charge) on C(3) and C(6), as expected from theoretical calculations.³⁰ A possible resonance form would contain no bond between C(3) and C(6), or even a bond between O(5) and O(7). The locations of bonding electrons and lone pair electrons are being investigated by low-temperature, high-resolution studies^{18,19} with the aim of analyzing the bonding in this C(3)–C(6) bond.

The central hydroxyl and carboxyl groups lie in a plane perpendicular to the plane of the backbone, C(2)–C(3)–C(4) (i.e., T13 is small). This coplanarity of the α -hydroxycarboxylic acid or α -hydroxycarboxylate grouping, which occurs in spite of the resultant close approach of O(5) to O(7), was noted originally in a study of tartrates³¹ and seems to be a general observation. Theoretical studies³² suggest that, for isolated molecules or ions (in vacuo), intramolecular hydrogen bonding between the hydroxyl hydrogen atom and one of the oxygen atoms of the central carboxyl group (i.e., between O(5) and O(7)) stabilizes this planar conformation. Such a hydrogen bond is observed experimentally in citric acid but is bifurcated,⁸ i.e., the hydroxyl hydrogen lies almost equidistant between the α -carboxyl group, O(5), and an oxygen atom of another molecule. In both anhydrous citric acid and the monohydrate the hydroxyl hydrogen atom lies out of the plane of the α -hydroxycarboxylic acid grouping (torsion angles H–O(7)–C(3)–C(6) = 37° (anhydrous) and 20° (monohydrate)). In metal chelates the cation lies near the position of this hydrogen atom, implying that the α -hydroxycarboxylate group is planar in citrates because this facilitates its action as a bidentate chelating group.

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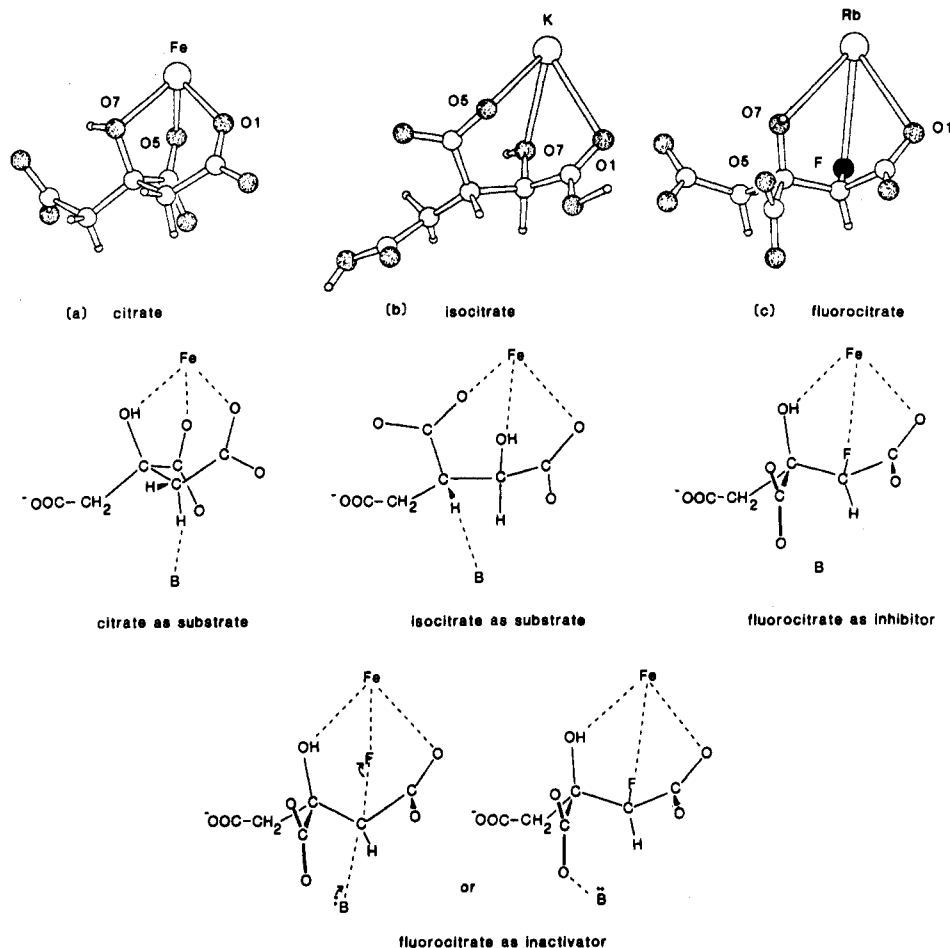


Figure 2. Tridentate chelation in (a) ferrous citrate, (b) potassium dihydrogen isocitrate, and (c) rubidium ammonium fluorocitrate. The manners in which these anions are involved in the suggested mechanism of action of aconitase are indicated for comparison.

However this coplanarity is not always possible for steric reasons. In sodium β -fluoropyruvate,³³ which crystallizes from water as a *gem*-diol, there are two hydroxyl groups α to the carboxyl group; these lie with HO-C-C-O⁻ torsion angles of 23.5 and 36.5°, respectively. A similar nonplanar α -hydroxycarboxylate group occurs in an acetylcitric anhydride derivative.³⁰

Each of the seven oxygen atoms of citrate can serve as a ligand to metals. This metal coordination can be monodentate, bidentate, or tridentate and, in the last two cases, generally involves the central hydroxyl and carboxyl groups (O(7) and O(5)). Magnesium,²¹ manganese,^{22,23} and ferrous²⁴ citrates form an isomorphous series of crystals (i.e., the structures are identical except for the presence and size of the metal ion) in which the citrate acts as a tridentate chelate (Figure 2a). The chelation of a complex antimony citrate²⁰ is shown in Figure 1b. NMR studies are consistent with similar structures for manganese and ferrous citrates in solution.³⁴

Citrate Analogues

Several analogues of citrate have been studied crystallographically. These are an isocitrate,³⁵ two hy-

droxycitrates,³⁶ two fluorocitrates,³⁷⁻³⁹ an anhydride derivative, and some *cis*-⁴⁰ and *trans*-aconitates.^{41,42} The latter two are dehydration products of citrate or isocitrate.

Isocitrate has the hydroxyl group on C(2) rather than C(3) and therefore contains two asymmetric carbon atoms resulting in four possible stereoisomers. The absolute configuration of the naturally occurring isomer, the anion of (+)-isocitric acid, has been established by X-ray crystallographic (as well as chemical and enzymatic) techniques. The structure of the potassium dihydrogen salt,³⁵ illustrated in Figure 2b, surprisingly shows that the central carboxyl group, not the terminal α -hydroxycarboxylic acid, is ionized. When hydroxyl groups are present on both C(2) and C(3), as in the hydroxycitrates, there are again four isomers, and their absolute configurations have also been established.^{36,44}

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Table I
Torsion Angles (deg) for Citrates in the Crystalline State^a

charge on cit	backbone								terminal carboxyl group							
	T1	T2	T3	T4	T5	T6	T7	T8	T9	T10	T11	T12	T13	T14	T15	T16
cit acid anhyd	-175	171	65	-69	8	-172	50	-130	-175	171	65	-69	8	-172	50	-130
cit acid monohyd	-174	180	61	-56	-63	117	48	-133	-174	180	61	-56	-63	117	48	-133
RbH ₂ cit	174	64	56	-176	-5	-177	-9	163	174	64	56	-176	-5	-177	-9	163
NaH ₂ cit	-56	-159	-174	-43	-42	140	-28	151	-56	-159	-174	-43	-42	140	-28	151
LiH ₂ cit	-56	-158	-176	-39	-41	142	-35	147	-56	-158	-176	-39	-41	142	-35	147
fenH ₂ cit	-171	175	68	-65	-77	102	68	-113	-171	175	68	-65	-77	102	68	-113
NH ₄ LiHcit	-172	170	68	-69	-44	138	52	-128	-172	170	68	-69	-44	138	52	-128
CaHcit	-176	69	65	-170	-66	113	-65	115	-176	69	65	-170	-66	113	-65	115
enHcit	-164	63	77	-177	-61	117	25	-156	-164	63	77	-177	-61	117	25	-156
Co(cit)(I)	-55	-167	-176	-49	84	-98	9	-168	-55	-167	-176	-49	84	-98	9	-168
Co(cit)(II)	-179	69	62	-171	-21	157	34	-147	-179	69	62	-171	-21	157	34	-147
Na ₃ cit	-177	164	64	-75	-29	154	-24	161	-177	164	64	-75	-29	154	-24	161
K ₃ cit	-174	160	65	-77	-61	120	76	-104	-174	160	65	-77	-61	120	76	-104
Sb(cit)(I)	-166	175	72	64	-67	114	24	-156	-166	175	72	64	-67	114	24	-156
Sb(cit)(II)	-66	-174	175	-57	79	-102	10	-165	-66	-174	175	-57	79	-102	10	-165
Mg(cit)	-170	-176	69	-54	72	-104	-14	166	-170	-176	69	-54	72	-104	-14	166
Mn(cit)	-170	-176	68	-55	72	-104	-11	169	-170	-176	68	-55	72	-104	-11	169
Fe(cit)	-170	-175	69	-53	75	-101	-14	167	-170	-175	69	-53	75	-101	-14	167
Ni(cit)(I)	-53	-174	-173	-57	-49	128	-29	152	-53	-174	-173	-57	-49	128	-29	152
Ni(cit)(II)	-173	175	68	-72	42	-136	-58	126	-173	175	68	-72	42	-136	-58	126
Ni(cit)(III)	167	175	52	-70	25	-152	-2	180	167	175	52	-70	25	-152	-2	180
Cu ₂ cit	-52	-179	-174	-61	-53	128	26	-158	-52	-179	-174	-61	-53	128	26	-158

charge on cit	central carboxyl group								O7 hydroxyl group							
	T9	T10	T11	T12	T13	T14	T15	T16	T9	T10	T11	T12	T13	T14	T15	T16
cit acid anhyd	-111	130	68	-51	12	-168	51	-158	-111	130	68	-51	12	-168	51	-158
cit acid monohyd	-116	121	63	-60	3	-178	62	-57	-116	121	63	-60	3	-178	62	-57
RbH ₂ cit	-139	99	43	-79	-15	167	68	-77	-139	99	43	-79	-15	167	68	-77
NaH ₂ cit	-102	138	77	-42	17	-164	68	77	-102	138	77	-42	17	-164	68	77
LiH ₂ cit	-101	138	75	-46	19	-165	67	78	-101	138	75	-46	19	-165	67	78
fenH ₂ cit	-118	122	62	-57	4	-175	57	-57	-118	122	62	-57	4	-175	57	-57
NH ₄ LiHcit	-126	117	54	-63	-4	176	50	-54	-126	117	54	-63	-4	176	50	-54
CaHcit	-137	101	45	-77	-16	166	60	-60	-137	101	45	-77	-16	166	60	-60
enHcit	-129	110	53	-68	8	-176	69	-53	-129	110	53	-68	8	-176	69	-53
Co(cit)(I)	-109	130	67	-55	10	173	60	-60	-109	130	67	-55	10	173	60	-60
Co(cit)(II)	-128	112	54	-66	-5	177	53	-53	-128	112	54	-66	-5	177	53	-53
Na ₃ cit	-137	103	44	-76	-18	163	46	-46	-137	103	44	-76	-18	163	46	-46
K ₃ cit	-127	114	56	-63	-6	177	42	-42	-127	114	56	-63	-6	177	42	-42
Sb(cit)(I)	-121	118	60	-60	-3	178	57	-57	-121	118	60	-60	-3	178	57	-57
Sb(cit)(II)	-116	123	63	-59	1	180	65	-65	-116	123	63	-59	1	180	65	-65
Mg(cit)	-127	113	54	-62	-4	177	64	-64	-127	113	54	-62	-4	177	64	-64
Mn(cit)	-121	118	59	-62	1	179	64	-64	-121	118	59	-62	1	179	64	-64
Fe(cit)	-124	115	57	-65	-2	179	65	-65	-124	115	57	-65	-2	179	65	-65
Ni(cit)(I)	-101	141	80	-38	17	162	70	-70	-101	141	80	-38	17	162	70	-70
Ni(cit)(II)	-90	157	85	-28	31	-154	51	-51	-90	157	85	-28	31	-154	51	-51
Ni(cit)(III)	-144	97	40	-79	-23	160	52	-52	-144	97	40	-79	-23	160	52	-52
Cu ₂ cit	-100	140	74	-45	19	-166	59	-59	-100	140	74	-45	19	-166	59	-59

^a The torsion angle (T) of a series of four atoms (1-2-3-4) is the angle of rotation needed to make the 1-2 and 3-4 bonds eclipse each other when viewed down the 2-3 direction. A positive torsion angle corresponds to a right-handed screw. Citric acid and citrate ions have been numbered so that T5, T7, and T13 are less than T6, T14, and T16, respectively, and so that T10 is arbitrarily positive. Note: T1 and T2 define the conformation of the backbone. If they are near 180°, the backbone is extended; if near ±60°, one end (never two) is swung around. T3 and T4 define the conformation of the central carboxyl group with respect to the backbone. They are dependent on values of T1 and T2 such that, of T1, T2, T3 and T4, two values are near ±60° and two near 180°. T5, T6, T7, and T8 define the conformations of the terminal carboxyl groups with respect to the backbone. Note that they may take almost any value. T9, T10, T11, and T12 define the conformation of the central carboxyl group with respect to the backbone. Note how little variation there is in each column. T13 and T14 define the planarity of the α-hydroxycarboxylic acid group. Roman numerals I, II, and III refer to different citrate ions in the same asymmetric unit of a unit cell in a given crystalline form of the citrate. Abbreviations: fen = fentanyl, en = ethylenediamine.

Table II
Average Distances and Angles in Citrates^a

Distances, Å	
C(1)-C(2) and C(4)-C(5)	1.511
C(2)-C(3) and C(3)-C(4)	1.536
C(3)-C(6)	1.544
C(3)-O(7)	1.433
C::O ionized carboxyl	1.257
C=O carboxyl group	1.215
C-OH carboxyl group	1.314
Angles, deg	
C(1)-C(2)-C(3) and C(3)-C(4)-C(5)	113.3°
C(2)-C(3)-C(4)	109.0
C(2)-C(3)-C(6) and C(4)-C(3)-C(6)	110.1
C(2)-C(3)-O(7) and C(4)-C(3)-O(7)	109.4
C(6)-C(3)-O(7)	108.8
ionized carboxyl group, O-C-O	123.9
C-C-O	118.0
nonionized carboxyl group, O=C-OH	123.6
C-C=O	123.4
C-C-OH	113.0

^a No correlation with ionization state could be found for any of these values. Values for RbH₂cit, LiH₂cit, Co(cit), and Sb(cit) and the tetraionized citrates have not been included in these averages because the precisions of the analyses are not high (esd ~ 0.02 Å, 1°). Estimated standard deviations for individual studies averaged here ranged from 0.002 to 0.008 Å and 0.1 to 0.5°.

The two fluorocitrates studied are the rubidium ammonium salt of the racemate of one pair of isomers^{37,38} and the (-)-benzylmethylamine salt of an ester of a resolved isomer.³⁹ The latter study, together with data on the absolute configuration of (-)-benzylmethylamine,⁴⁵ established the absolute configuration of this isomer and of its naturally occurring enantiomer. One of the major points of interest for the metal salt of the racemate was the fact that the fluorine atom took part in the rubidium coordination sphere, excluding the central carboxyl group from this sphere (shown in Figure 2c). It was also found that the conformation of the fluorocitrate was very similar to that of the analogous hydroxycitrate. No evidence of F-H-O hydrogen bonds was found in any of the fluorocitrates studied by X-ray techniques.

Citrate Enzymes

The structural features of the citrate ion that have just been described can be used to determine the stereochemistry of the interaction of citrate and its analogues with enzymes. Such studies have led to the formulation of hypotheses on the mechanisms of the enzymes involved. The enzymes considered here are aconitase, which can cause both isomerization and dehydration of citrate, citrate synthase, which catalyzes the stereospecific formation of citrate from acetyl-CoA and oxaloacetate, and the two citrate lyases, which catalyze the cleavage of citrate to give acetate and oxaloacetate. All are active in major metabolic pathways in cells.

Aconitase may be considered as both an isomerase and a hydratase because it interconverts three substrates—citrate, isocitrate, and *cis*-aconitate.⁴⁶ It is the second enzyme in the Krebs cycle, and it catalyzes the removal of both a hydrogen atom and hydroxyl group

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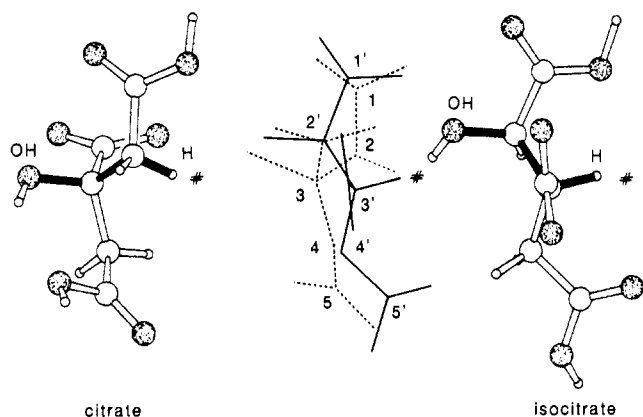


Figure 3. Comparison of the conformations of the sodium dihydrogen citrate and potassium dihydrogen isocitrate anions. These are viewed with respect to the best plane through the terminal carboxyl groups. The bonds involved in the hydration or dehydration process are black and the hydrogen atom abstracted from each in the aconitase reaction is indicated by #.

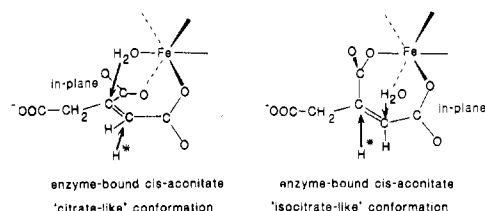


Figure 4. Conformations of enzyme-bound *cis*-aconitate showing "citrate-like" and "isocitrate-like" conformations. Note that a water molecule and the central carboxyl group have interchanged positions on the coordination sphere.

from citrate. The hydrogen atom of citrate that is removed is the *pro*-2*S*,3*R* hydrogen atom⁴⁷ (the *pro*-2*S* hydrogen atom on the *pro*-3*R* carboxymethyl group, asterisked in III). This information, combined with the known absolute configuration of isocitrate formed,⁴³ indicates that a *trans* addition of the elements of water to *cis*-aconitate has occurred. Addition to the double bond in one way gives citrate, in the opposite way gives isocitrate.

The hydrogen atom which is abstracted from either citrate or isocitrate by the enzyme aconitase is retained by the enzyme⁴⁸ and is either added back again to the same face of the double bond of *cis*-aconitate to give citrate or is added to the opposite face to give isocitrate. Thus, as pointed out by Ogston,⁴⁹ the plane of the double bond of *cis*-aconitate must rotate during the enzymatic reaction. Otherwise, the hydrogen atom retained by a side chain of the enzyme could not reach the other side of this plane without some exchange with the environment in the process.

Information from X-ray studies has been used to analyze this problem.⁵⁰ When the shapes of citrate in sodium dihydrogen citrate and isocitrate in potassium dihydrogen isocitrate are suitably compared, as shown in Figure 3, on the assumption that the enzyme binds the ends of the two carboxymethyl groups, the hydrogen atom abstracted by the enzyme is similarly located for both anions. Furthermore the metal chelation indicates that, since the metal position is fairly constant when

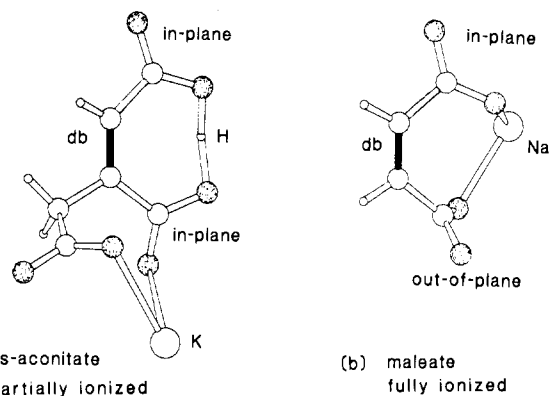


Figure 5. (a) Dipotassium *cis*-aconitate hydrogen (not fully ionized) showing that both carboxyl groups lie in the plane of the double bond (db) and that there is a tight, symmetrical internal hydrogen bond between them. The metal ion binds to the central and other terminal carboxyl group. (b) Disodium maleate (fully ionized) showing one carboxyl group in the plane of the double bond and the other carboxyl group out of this plane. The metal chelation is similar to that proposed for aconitase binding.

the abstracted hydrogen atom position is fixed, chelation (to ferrous iron) may be part of the mechanism. In the "ferrous wheel" mechanism⁵⁰ (shown in part in Figure 4) it is proposed that all three carboxyl groups of citrate are held on the enzyme (since none of the three monoesters of citric acid are active)⁵¹ and the substrates (citrate or isocitrate) chelate to the ferrous iron via two carboxyl groups and a hydroxyl group. Removal of the elements of water gives enzyme-bound *cis*-aconitase in one of two conformations referred to as "citrate-like" and "isocitrate-like", respectively (Figure 4). These two conformers differ by the interchange of the central carboxyl group and a water molecule on the coordination octahedron of the ferrous ion. The hydrogen atom that is abstracted is added again from the same side chain (such as glutamate or aspartate) in the active site of the enzyme to the enzyme-bound *cis*-aconitate intermediate to give either hydroxy acid again. The anion conformations and the chelation proposed are the same as those found experimentally and illustrated in Figure 2a,b. Side chains, such as arginine, would provide likely groups for attachment of terminal carboxyl groups of citrate to the enzyme.⁵²⁻⁵⁴ The addition of water occurs in a *trans* fashion perpendicular to the plane of the double bond of the *cis*-aconitate intermediate; this plane rotates 90° as shown in Figure 4, and predicted earlier.⁴⁹

The mechanism has some symmetry in that one carboxyl group must be coplanar with the double bond of the enzyme-bound *cis*-aconitate, while the other is rotated out of this plane. In the formation of both citrate and isocitrate, the hydroxyl group adds to the carbon atom adjacent to the carboxyl group that is coplanar with the double bond and the abstracted hydrogen atom is added in a *trans* fashion to the other carbon atom. After this feature had been proposed,⁵⁰ the structures of two diionized maleates were reported;^{55,56} they showed that when both carboxyl groups are

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ionized, one is coplanar with the double bond and the other is out of the plane, as shown in Figure 5. *cis*-Aconitate may be considered to be a derivative of maleate with one hydrogen atom replaced by a $-\text{CH}_2\text{COOH}$ group and therefore it is an adequate model for enzyme-bound *cis*-aconitate. In both *cis*-aconitate and maleate a short internal hydrogen bond is formed between the two carboxyl groups if only one carboxyl group is ionized.⁴⁰ NMR studies of an active aconitase-ferrous-citrate bridge complex and an inactive aconitase-manganous-citrate bridge complex⁵⁷ gave metal-to-citrate distances in agreement with the above-proposed mechanism involving chelation.⁵⁰

The enzyme can distinguish between the two $-\text{CH}_2\text{COO}^-$ groups of citrate. When citrate is bound to aconitase, if O(7) and O(5) are "recognition points", the binding is asymmetric since the backbone lies through C(3) which is attached to O(7) but not O(5). Therefore, when citrate binds the "wrong way", hydrogen atoms on C(2) or C(4) are not in the correct position to be abstracted by a fixed group in the active site of the enzyme. This phenomenon has been described by Ogston as "three-point attachment",^{58,59} a principle first applied to the interaction of asymmetric drugs with receptors⁶⁰ but not extended to prochiral molecules until Ogston's work. The three points are O(7), O(5), and the appropriate hydrogen atom on C(2). An example of such differentiation by "three-point" attachment has been studied crystallographically and spectroscopically.⁶¹

Gawron⁶² reported that the spectrum of aconitase was characteristic of a non-heme iron protein, and, more recently, Beinert⁶³ found that aconitase is a high-potential iron protein containing an iron-sulfur center. It is not clear whether the role of this iron-sulfur group is structural, catalytic, or regulatory. There is, however, a lack of correlation of the activity of aconitase with the extent of irreversible damage to this iron-sulfur center, as manifested by the exposure of the iron to water.⁶⁴

One good way to find out how aconitase works and to establish the role of the iron-sulfur group is to determine the crystal structure of the enzyme. Unfortunately aconitase is an unstable enzyme and can function only in a reducing environment, and this has caused problems in preparing crystals. The cell dimensions for some twinned crystals have been reported⁶⁵ as $a = 174.1$, $b = 72.0$, $c = 72.8$ Å, and further studies are in progress in the laboratories of Gawron, Sax, and Glusker to try to obtain crystals of a higher quality for a complete X-ray structure determination of the pig

heart enzyme. The enzymes from yeast⁶⁶ and *Candida lipolytica*⁶⁷ have also been purified and the formation of crystals reported.

Much more structural information is available for citrate synthase. This enzyme regulates entry into the Krebs cycle and causes formation of citrate from oxaloacetate and acetyl-CoA. Crystallographic studies on the pig heart enzyme⁶⁸ and the chicken heart enzyme⁶⁹ (two forms, one grown with ATP present) and for the higher molecular weight enzyme from *E. coli*⁷⁰ have been made. The structure of the pig heart enzyme, at 3.5-Å resolution,⁶⁸ has revealed a structure rich in α -helices but with a disordered region (probably the N-terminal end). Further studies are in progress in Huber's laboratory.

Effects of Citrate Analogues on Citrate Enzymes

The citrate analogues, particularly fluorocitrate and hydroxycitrate, are excellent probes for the active sites of citrate enzymes, particularly since each analogue has four stereoisomers. Peters and co-workers^{71,72} showed that the toxicity to animals of fluoroacetate, found in certain plants, is due to its enzymatic conversion, by citrate synthase, to fluorocitrate. The fluorocitrate acts as an inhibitor of aconitase. As a result, energy production is affected, and citrate accumulates and binds calcium, thereby making it unavailable. Kun⁷³ suggested that the toxicity of fluorocitrate is caused by its inhibition of the tricarboxylate carrier protein.

Kun⁷⁴ showed that only one isomer is a strong inhibitor and inactivator of aconitase. The absolute configuration of this isomer^{37,39} was shown, by X-ray studies, to be *2R,3R*, as illustrated in Figure 2c. Thus citrate synthase can act on both fluoroacetyl-CoA and acetyl-CoA in the same manner. Thus the isomer of fluorocitrate that inhibits aconitase has the fluorine atom on the acetate-derived end (*pro-S*) of modified citrate, whereas aconitase acts on the other end (oxaloacetate-derived, *pro-R*) of citrate. The inhibitory isomer is initially a linear competitive inhibitor of aconitase, indicating that the fluorocitrate competes with citrate in the active site but this action is followed by a time-dependent inactivation.³⁷ Presumably, then, the fluorocitrate chelates enzyme-bound metal in the same manner that it chelates rubidium in its salt,^{37,38} as shown in Figure 2c. This type of binding cannot be productive, however, because there is no hydrogen atom available for abstraction. Also, the central carboxyl group sticks up toward the proton-abstracting site. As a result, some interaction with the active site, such as hydrogen bonding or alkylation via the central carboxyl group, occurs so that productive binding of substrate

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can no longer take place and therefore the enzyme is inactivated.

Another set of interesting analogues are the hydroxycitrates. Two of the four isomers occur naturally in plants.⁷⁵ Their interactions with citrate enzymes are different from those of the fluorocitrates. None of the four isomers of hydroxycitrate is a substrate for citrate synthase, even though one of the analogous fluorocitrates is a substrate. However, the hydroxycitrates bind to the citrate lyases (bacterial citrate lyase and cytosolic ATP citrate lyase) and behave as substrates or inhibitors. The shapes of the active sites of these lyase enzymes can be "mapped" by a consideration of the shapes of the hydroxycitrates, and some explanation of the catalytic and binding activities provided.^{36,76}

Each hydroxycitrate isomer has two hydroxyl groups and three carboxyl groups from which one to three of these groups may chelate to the enzyme-bound magnesium ion. One isomer of hydroxycitrate ((2*S*)-(p*n*_{cit})-) is inactive in bacterial citrate lyase, presumably because it binds in a "wrong" or unproductive way. Bacterial citrate lyase is active only when it is "activated" by acetylation. When the activated enzyme binds citrate, acetate is lost, but, as a result of the catalysis, cleavage of this citrate to acetate occurs, leaving regenerated "activated" enzyme. However, if hydroxycitrate binds in an unproductive manner, the acetyl group is lost; but activated enzyme is not regenerated as the intact hydroxycitrate leaves. Unproductive binding can therefore, in itself, be inhibitory.⁷⁶ ATP citrate lyase (citrate cleavage enzyme) is very strongly inactivated by one isomer⁷⁷ ((4*S*)-(p*n*_{cit})-) and, presumably, as this isomer binds, one carboxyl group sticks up and interacts with a protein side chain in the active site. The exact nature of this interaction is not known, but it could be similar to the interaction proposed for fluorocitrate in aconitase. This isomer of hydroxycitrate is of particular interest because its inhibition of ATP citrate lyase results in an inhibition of cholesterol and triglyceride

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syntheses in vivo.⁷⁸ Thus it may have important medicinal uses.

Other analogues are also good probes for citrate enzyme activity. X-ray structural studies on the methylcitrates (substituted on the methylene group), some of which occur naturally,⁷⁹⁻⁸¹ and on a nitrocitrate which is a very powerful inhibitor of aconitase and may mimic the transition-state geometry of citrate,^{82,83} have been initiated.

Conclusions

Studies of the mechanisms of enzyme-catalyzed processes involve many chemical and biochemical studies. One very important part of our understanding of enzyme activity must come from a detailed knowledge of the three-dimensional structures of both the enzyme and its substrates and inhibitors. The stereochemistry of the active site is then known. This information can then be combined with NMR, spectroscopic, binding, and catalytic activity studies to give a complete picture of the catalytic process. In this Account the usefulness of detailed stereochemical studies of enzyme substrates and inhibitors in suggesting mechanisms of action that can be tested has been emphasized.

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