Effects of Salts and Organic Solvents on the Activity of Citrate Synthase*

(Jang-Yen Wu‡ and Jen Tsi Yang)

From the Department of Biochemistry and Biophysics and Cardiovascular Research Institute, University of California, San Francisco, California 94122

SUMMARY

The enzymatic activity of citrate synthase from pig heart can be inhibited by various ions. The order of inhibitory effect of monovalent cations is Li⁺ > K⁺ > Na⁺ > Cs⁺ and that of anions is SCN⁻ > Cl⁻ > tartrate. Divalent cations such as Ca²⁺ and Mg²⁺ are much more effective than monovalent cations, but thiocyanate is as effective as divalent cations. ATP is also a potent inhibitor, but dATP has no effect. The inhibition by mono- and divalent cations and ATP can be overcome by an increase in the concentration of one of the substrates, acetyl coenzyme A, but not the other, oxalacetate.

Citrate synthase catalyzes the reaction of acetyl coenzyme A and oxalacetate to form citrico acid. It does not require any cofactor, metallic ion, or activator for its optimum activity. A number of compounds have been shown to inhibit the activity of citrate synthase. To the first category belong the end products of the tricarboxylic acid cycle initiated by citrate synthase such as ATP (1–3), palmityl-CoA (4–8), and other nucleotides (2) which are classified as feedback inhibitors. The second category includes substrate analogues such as citryl-CoA (9), 8-monofluorooxalacetate (10), and polyphosphates (11), which are competitive inhibitors with both acetyl-CoA and oxalacetate or with acetyl-CoA alone. A third class of inhibitors is divalent cations such as Mg²⁺, Ca²⁺, and Mn²⁺ (3). This communication will present results of inhibition by monovalent cations and anions. Furthermore, the inhibitory effects of these ions are approximately parallel to their lyotropic effects.

EXPERIMENTAL PROCEDURE

Materials—Citrate synthase from pig heart, purchased from Boehringer Mannheim Corporation, was a crystalline suspension in 2.2 M ammonium sulfate (pH about 7). Its specific activity was approximately 70 units per mg.

* This work was aided by Grant GM-10880, HE-05285, and GM-K3-3441 from the United States Public Health Service. Presented orally at the Pacific Slope Biochemical Conference, Santa Barbara, California, September, 1968.
‡ Present address, Molecular Biology Institute, UCLA, Los Angeles, California 90024.

Acetyl-CoA, oxalacetate, 5,5′-dithiobis(2-nitrobenzoic acid), ATP, ADP, AMP, and dATP were purchased from Calbiochem. Potassium salts of phosphate, chloride, thiocyanate, and tartrate and the chlorides of lithium, sodium, and calcium were obtained from Allied Chemical and Dye Corporation. Cesium chloride was obtained from Harshaw Chemical Company, Cleveland, Ohio. Tris from Sigma, magnesium acetate from Fisher, dioxyane from Matheson, Coleman and Bell, Division of the Matheson Company, and ethanol from Commercial Solvent Corporation, Terre Haute, Indiana.

Method—The citrate synthase solution was prepared by dialyzing the protein suspensions against 0.1 M Tris buffer (pH 8.2) or 0.1 M phosphate buffer (pH 7.4) at 4°, followed by centrifugation to remove any precipitate. The concentration of the enzyme solution was determined spectrophotometrically with the use of $E_{1%}^{1cm} = 15.0$ at 280 nm. Details have been described previously (12).

The enzyme was assayed at pH 8.2 with the method of Srere, Brazil, and Gonen (13). It is based on the reaction of CoASH with 5,5′-dithiobis(2-nitrobenzoic acid) to produce a mercaptoion which absorbs at 412 nm. The rate of CoASH formation (from acetyl-CoA and oxalacetate in the presence of the enzyme) is followed spectrophotometrically. These measurements were made in a Beckman model DU spectrophotometer with the attachment of a Gilford 2000 multiple sample absorbance recorder. The maximum activity of citrate synthase was 80 units.

RESULTS

Effect of Cations—Fig. 1 shows the percentage of inhibition of the enzymatic activity as a function of monovalent cation chlorides. The enzymatic activity in 0.1 M phosphate buffer (pH 7.4) or 0.1 M Tris buffer (pH 8.2) was taken as 100%. The most effective inhibitor was Li⁺, followed by K⁺, Na⁺, and Cs⁺. The concentrations required for 50% inhibition by the corresponding chlorides were 0.21, 0.26, 0.32, and 0.42 M, respectively.

The divalent cations such as Ca²⁺ and Mg²⁺ are far more effective than the monovalent ones. This is illustrated in Fig. 2, where the ionic strength of calcium chloride and magnesium acetate required for 50% inhibition was only 0.042 and 0.056, respectively. Figs. 3 and 4 show that the inhibitory effects of salts such as magnesium acetate (ionic strength 0.06) and lithium chloride (ionic strength 0.15) could be completely overcome by an increase in the concentration of one of the substrates, acetyl-CoA, but not the other, oxalacetate.
Inhibition of the enzymatic activity of citrate synthase by monovalent cation chlorides. The standard assay medium contained $5 \times 10^{-4} \text{M}$ acetyl-CoA, $6.25 \times 10^{-4} \text{M}$ oxalacetate, $5 \times 10^{-4} \text{M}$ 5,5'-dithiobis(2-nitrobenzoic acid), and $2.0 \times 10^{-4} \text{g}$ of citrate synthase. All salts were dissolved in 0.1 M potassium phosphate buffer (pH 7.4). In all enzyme assays, the medium was the same unless otherwise stated.

**Effect of Anions**—Fig. 5 shows the percentage of inhibition as a function of anion concentration. Potassium thiocyanate was the most effective inhibitor, followed by potassium chloride and potassium tartrate. The corresponding ionic strengths required for 50% inhibition were 0.055, 0.26, and 0.63, respectively. Note also that thiocyanate is as effective as the divalent cations as an inhibitor of citrate synthase.

**Effect of Buffer**—In order to determine whether the concentration of buffer used in this study could have an inhibitory effect, we measured the enzymatic activity of citrate synthase in various concentrations of the buffers, taking again the activities in 0.1 M buffer as the reference state (100%). Fig. 6 shows that the maximum activity of citrate synthase in Tris buffer (pH 8.2) and phosphate buffer (pH 7.4) occurs at a concentration of 0.03 to 0.1 M and 0.05 M, respectively. Since 0.1 M buffers were used in the standard assay, we conclude that these concentrations are not inhibitory.

**Fig. 1.** Inhibition of the enzymatic activity of citrate synthase by monovalent cation chlorides. The standard assay medium contained $5 \times 10^{-4} \text{M}$ acetyl-CoA, $6.25 \times 10^{-4} \text{M}$ oxalacetate, $5 \times 10^{-4} \text{M}$ 5,5'-dithiobis(2-nitrobenzoic acid), and $2.0 \times 10^{-4} \text{g}$ of citrate synthase. All salts were dissolved in 0.1 M potassium phosphate buffer (pH 7.4). In all enzyme assays, the medium was the same unless otherwise stated.

**Fig. 2.** Inhibition of the enzymatic activity of citrate synthase by divalent cations. Calcium chloride and magnesium acetate were dissolved in 0.1 M Tris buffer (pH 8.2). Potassium thiocyanate is included for comparison (see Fig. 5).

**Fig. 3.** Dependence of the inhibitory effect of divalent cation upon the concentration of substrates. The ionic strength of $\text{Mg(CH}_2\text{COO)}_2$ was 0.06.

**Fig. 4.** Dependence of the inhibitory effect of monovalent cation upon the concentration of acetyl-CoA. The ionic strength of LiCl was 0.15.

**Fig. 5.** Inhibition of the enzymatic activity of citrate synthase by the anions of potassium salts.
I, I, I

CONCENTRATION (M)

FIG. 6. Dependence of the enzymatic activity of citrate synthase upon the concentration of Tris buffer (pH 8.2) and phosphate buffer (pH 7.4).

0.02 0.04 0.06 0.08 0.10

ACTIVITY

TRIS

PHOSPHATE

FIG. 7. Dependence of the enzymatic activity of citrate synthase upon the concentration of organic solvents.

0.0 0.1 0.2 0.3 0.4 0.5

CONCENTRATION (M)

Effect of Organic Solvents—Ethanol and dioxane were used to study the effect of nonpolar solvent on the activity of citrate synthase. Fig. 7 shows a plot of percentage of activity against the concentration of the organic solvent (expressed in terms of volume of ethanol or dioxane per volume of 0.1 M phosphate buffer, pH 7.4). At very low concentration of ethanol or dioxane there was actually a slight activation. This was followed by a marked decrease in activity with increasing concentration of ethanol or dioxane. The 50% inhibition was found to be at 16% dioxane and 19% ethanol.

Effect of Nucleotides—Fig. 8 includes plots of the percentage of inhibition against the concentration of nucleotides. As observed by Hathaway and Atkinson for yeast citrate synthase (2), the effectiveness of the inhibition in decreasing order was ATP > ADP > AMP. The inhibition by ATP, however, could be overcome by an increase in the concentration of acetyl-CoA, but not oxalacetate (Fig. 9). dATP did not inhibit the activity of citrate synthase even at a concentration of 5 mM. At this concentration ATP caused about 60% inhibition.

FIG. 8. Dependence of the enzymatic activity of citrate synthase upon the concentration of nucleotides.

FIG. 9. Dependence of the inhibitory effect of ATP upon the concentration of acetyl-CoA.

Discussion

This study, the inhibitory effect of various salts on the activity of citrate synthase can largely be attributed to the cations and anions used rather than to the buffers.

Effect of Organic Solvents—Ethanol and dioxane were used to study the effect of nonpolar solvent on the activity of citrate synthase. At very low concentration of ethanol or dioxane there was actually a slight activation. This was followed by a marked decrease in activity with increasing concentration of ethanol or dioxane. The 50% inhibition was found to be at 16% dioxane and 19% ethanol.

Effect of Nucleotides—Fig. 8 includes plots of the percentage of inhibition against the concentration of nucleotides. As observed by Hathaway and Atkinson for yeast citrate synthase (2), the effectiveness of the inhibition in decreasing order was ATP > ADP > AMP. The inhibition by ATP, however, could be overcome by an increase in the concentration of acetyl-CoA, but not oxalacetate (Fig. 9). dATP did not inhibit the activity of citrate synthase even at a concentration of 5 mM. At this concentration ATP caused about 60% inhibition.

Discussion

The order of the inhibitory effect of various cations and anions fall roughly into the Hofmeister series (14) for the salting-in of proteins. von Hippel and Schleich (15) showed that ions that effectively stabilize (or destabilize) the conformations of macromolecules are also effective salting out (or salting in) agents for various diverse macromolecules such as DNA, collagen, and ribonuclease. The order of decreasing effectiveness as salting-in or denaturing agents is Li⁺ > Na⁺ > K⁺ > Rb⁺ > Cs⁺, Ca²⁺ > Mg²⁺, and SCN⁻ > I⁻ > NO₃⁻, Br⁻ > Cl⁻ > CH₃COO⁻ > SO₄²⁻ > tartrate. von Hippel and Schleich (15) further pointed out that this generality of the effects of ions can also be applicable to the protein subunit association dissociation equilibria, macroscopic phase transitions, enzymatic activities, and kinetics of macromolecular folding unfolding reactions.

In a previous communication (12) we reported that the hydrodynamic properties of citrate synthase in high salt solutions, for instance, 2 M KCl, did not change significantly, although the enzymatic activity was completely inhibited (Fig. 1). The sedimentation coefficient at a protein concentration of 5.6 mg per ml...
was 4.3 S in 2 M KCl as compared with 5.6 S for citrate synthase at 6 mg per ml in 0.1 M Tris buffer (pH 8.2). The corresponding intrinsic viscosity increased slightly from 3.95 to 4.50 ml per g. Thus, the loss of enzymatic activity in this case could not be attributed to the dissociation or association of citrate synthase. (The molecular weight in 2 M KCl, based on the Scheraga-Mandelkern equation (16), was estimated to be about 91,000.) Nevertheless, the hydrodynamic properties in 2 M KCl did indicate some change of the over-all shape of the protein molecule. Previously (12), we also reported that the optical rotatory dispersion and circular dichroism spectra of citrate synthase in 2 M KCl were similar to those of citrate synthase in 0.1 M Tris buffer. That is, the optical rotatory dispersion displayed a 233 nm trough and the circular dichroism a double minimum at 222 and 210 nm, both characteristic of an a-helix. Their magnitude, however, did reduce in the presence of high salt. For example, the reduced mean residue rotation, \([\phi]_{233}\), was -6,100 instead of -7,150, and the mean residue ellipticities, \([\phi]_{200}\) and \([\phi]_{222}\), were -15,000 and -19,000 instead of -20,000 and -21,000, respectively. All these indicated that the helical content of citrate synthase in high salt solution seemed to be slightly reduced, but they also ruled out any drastic change in conformation such as the complete unfolding of the protein molecule. This conclusion is also consistent with the findings of the hydrodynamic properties. The KCl-treated citrate synthase was enzymatically active when the salt concentration was reduced as in a standard assay, thus suggesting a reversible inhibition.

Our results also indicated that citrate synthase was inhibited by the increase in ionic strength of the buffer solutions (Fig 6). Furthermore, the inhibition of enzymatic activity by various salts was diminished by a gradual increase in the concentration of one of the substrates, acetyl-CoA, but not the other substrate, oxalacetate. Thus, the inhibition is competitive and the ions interfere with the binding affinity between citrate synthase and acetyl-CoA. The inhibition by various ions can then be explained in terms of the ion shielding effect in addition to the conformational change of the protein molecule in the presence of salts. In the monovalent cation series, Li\(^+\) is actually the most hydrated ion and Cs\(^+\) the least. Electrostatic interaction will bring the positively charged hydrated ion to the negatively charged site on the protein molecule. Thus, the most hydrated anion, SCN\(^-\), is the most effective inhibitor in the anionic series.

The inhibition of the enzymatic activity of citrate synthase by divalent cations such as Mg\(^{2+}\) and Ca\(^{2+}\) is much more effective than that of the monovalent cations. Kosicki and Lee (3) interpreted this as the result of chelate formation between the divalent cations and acetyl-CoA, very likely at the polyphosphate chain of the latter. Note, however, that the monovalent anion, SCN\(^-\), does not chelate the polyphosphate chain, but its inhibitory effect on citrate synthase is comparable to that of the divalent cations. Thus, it is difficult to decide whether the inhibition by divalent cations is due to (a) the chelate formation between the cation and substrate or the negatively charged site on the protein molecule or (b) the general ionic effect, or both.

We have confirmed that ATP is a potent inhibitor for citrate synthase, but dATP has no effect on the enzymatic activity. Ts'o, Rapaport, and Bollum (17) suggested that the 2'-hydroxyl group of ribose forms an intramolecular hydrogen bond with the purine base. Perhaps this hydrogen bond is essential to maintain a proper conformation of the nucleotide so that it can bind with the enzyme molecule.

Organic solvents such as dioxane and ethanol can also reduce the enzymatic activity of citrate synthase drastically, even though the optical and hydrodynamic properties indicated otherwise. For example, the sedimentation coefficient of citrate synthase in 10% dioxane (v/v) was 5.6 S at a protein concentration of about 8 mg per ml, which was not significantly different from that of citrate synthase in 0.1 M Tris buffer (12). This ruled out the possibility of dissociation or association. As in the case of 2 M KCl, the optical rotatory dispersion and circular dichroism spectra of citrate synthase in 10% dioxane resembled those in 0.1 M Tris buffer. But the magnitude of \([\phi]_{233}\) reduced to -5,600, \([\phi]_{200}\) to -16,700, and \([\phi]_{222}\) to -18,000, again suggesting a reduction in the helical content of citrate synthase in the presence of dioxane. The dioxane-treated citrate synthase was again enzymatically active in a standard assay. Note that the enzymatic activity was slightly increased at very low concentration of dioxane or ethanol (Fig 7). At relatively high concentration of these organic solvents, the enzyme was drastically inhibited, as indicated above. This is most likely due to the change in conformation of the protein molecule under these conditions.

REFERENCES
Effects of Salts and Organic Solvents on the Activity of Citrate Synthase
Jang-Yen Wu and Jen Tsi Yang


Access the most updated version of this article at http://www.jbc.org/content/245/14/3561

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 0 references, 0 of which can be accessed free at http://www.jbc.org/content/245/14/3561.full.html#ref-list-1