Citrate Synthase

A REGULATORY ENZYME FROM RHODOPSEUDOMONAS CAPSULATA*

LEON EIDELS† AND JACK PREISS

From the Department of Biochemistry and Biophysics, University of California, Davis, California 95616

SUMMARY

Citrate synthase (citrate oxaloacetate-lyase (CoA-acetylating), EC 4.1.3.7) is very active in *Rhodopseudomonas capsulata*, a nonsulfur purple bacterium. This activity varies greatly from carbon source to carbon source. The highest specific activity for this enzyme was obtained in acetate and acetate-bicarbonate-grown cells (1.7 to 1.8 μmoles per mg of protein-min). Citrate synthase activity was 2-fold greater in cells grown aerobically in the dark than in cells grown semiaerobically in the light, when either malate or pyruvate was the carbon source. The specific activities of this enzyme from glucose dark-grown and light-grown cells, and from pyruvate dark-grown cells were similar, and they were about one-half the specific activity of the enzyme from acetate-grown cells.

Citrate synthase in this organism was found to be a regulatory enzyme. DPNH was a strong inhibitor of the enzyme and AMP was found to relieve this inhibition. DPNH, at 0.1 mM, increased the S0.5 value and the n value (Hill constant) for acetyl-CoA. It also increased the S0.5 value but did not change the n value for oxaloacetate. Increasing amounts of DPNH increased the n value for AMP, the "de-inhibitor". AMP, 0.46 mM, in the presence of DPNH, restored the S0.5 and the n values for acetyl-CoA to those obtained in the absence of effector molecules. AMP (0.46 mM) alone did not affect these values. Thus, AMP acts strictly as a deinhibitor of the DPNH effect with respect to acetyl-CoA. AMP, in the presence or absence of DPNH, decreased the S0.5 value and the n value for oxaloacetate acting here both as a deinhibitor and as an activator. These results suggest that citrate synthase activity in *R. capsulata* is modulated both by the "energy charge" and the "reducing state" of the cell.

* This research was supported in part by United States Public Health Service Grant AI 05520 from the National Institutes of Health.
† Present address, Department of Microbiology, University of Connecticut School of Medicine, Farmington, Connecticut 06032.

At present there is no detailed report on the kinetics of a citrate synthase that is inhibited by DPNH and whose inhibition is relieved by AMP.

The present communication reports the levels of citrate synthase from *R. capsulata* grown under a variety of conditions, as well as the detailed kinetic properties of this enzyme which is specifically inhibited by DPNH and deinhibited by AMP. The

\[ \text{(CoA-acetylating), EC 4.1.3.7) in a large number of bacteria has been reported (2). The enzymes from bovine heart, bovine liver, and *Escherichia coli* were reported by Jangaard, Unkeless, and Atkinson (3) to be inhibited by ATP. However, Weitzman (4) reported that citrate synthase from *E. coli* was rather insensitive to ATP inhibition but was quite sensitive to DPNH inhibition (0.1 mM produced 90% inhibition). Subsequently, he reported that inhibition by 1 mM DPNH was completely overcome by 0.8 mM AMP (5). More recently, Weitzman and Jones (2) studied the effect of DPNH on the citrate synthase activity of a variety of bacteria. They found that the enzyme from gram-positive organisms was not inhibited by DPNH while the enzyme from gram-negative organisms was. Moreover, the gram-negative organisms could be subdivided into two groups; in one group (strict aerobes) the inhibition of citrate synthase by DPNH (0.8 mM) was relieved by AMP (0.5 mM) while in the other group (facultative anaerobes) AMP had no effect.

*Rhodopseudomonas capsulata* is a photosynthetic, nonsulfur, purple bacterium (*Aithiorrhodaceae*) capable of photoautotrophic and photoheterotrophic growth. It is also considered to be a facultative aerobe and can grow in the dark in the presence of organic compounds and oxygen (6). *R. capsulata* has traditionally been grown both in the light and in the dark in a minimal salts medium with malate as the sole carbon source (7). It has also been grown on glucose or pyruvate both in the light and in the dark, on acetate or acetate-bicarbonate in the dark, and on hydrogen-carbon dioxide in the light (7, 8). Recently we have shown that this organism metabolizes glucose almost exclusively via an inducible Entner-Doudoroff pathway. Moreover, very low levels of phosphofructokinase and 6-phosphogluconate dehydrogenase were found in *R. capsulata* suggesting that both the Embden-Meyerhof pathway and the hexosemonophosphate shunt are of minor physiological importance in this organism. Although the presence of citrate synthase in *R. capsulata* has been reported (9), the regulation of the enzyme in this organism had not been studied.

The present communication reports the levels of citrate synthase from *R. capsulata* grown under a variety of conditions, as well as the detailed kinetic properties of this enzyme which is specifically inhibited by DPNH and deinhibited by AMP. The

1. L. Eidels and J. Preiss, unpublished results.
significance of these observations with respect to the regulation of the flow of carbon in this organism is discussed.

**EXPERIMENTAL PROCEDURE**

The organism was obtained from Dr. H. Gest of the Department of Bacteriology, Indiana University, and was maintained in the laboratory as stab cultures (10). Cultures were grown semiaerobically in the light or aerobically in the dark in New Brunswick fermentors in 12 liters of the synthetic medium of Ormerod, Ormerod, and Gest (7). The cultures were grown at 31 to 33° until they reached stationary phase, at which time they were harvested at 4° in a Sharples continuous flow centrifuge. The cells were stored at 12°.

Grade I protamine sulfate, Tris, DPN+, and DPNH were obtained from Sigma. TPN+, acetyl-CoA, and HS-CoA2 were obtained from P-L Biochemicals. Oxalacetate, ADP, and ATP were obtained from Calbiochem. AMP was obtained from Schwarz BioResearch, and DTNB was obtained from Aldrich.

Preparation of Cell-free Extract—Acetate-grown dark-grown cells, 10 g, were resuspended in 100 ml of 0.05 M Tris-HCl (pH 8.0) buffer and exposed to sonic oscillation with a Biosonik III in two 50-ml fractions for a total of 5 min with intermittent cooling in order to keep the temperature below 15°. Unbroken cells and cell debris were removed by centrifugation at 48,200 x g for 30 min in a Sorvall centrifuge. Subsequent steps were carried out at 4°, unless otherwise specified.

Heat Treatment—The enzyme is relatively heat stable, and can be heated to 60° for 5 min with very little loss in activity. In the presence of AMP, a deinhibitor of the enzyme, it can be heated to 60° with no loss in activity. The Sorvall supernatant fluid was made 0.01 M Tris-HCl with respect to AMP, transferred to a 500-ml Erlenmeyer flask, and heated to 56° in a 60° water bath (12 liters). After reaching 56° (about 5 min), the solution was kept at this temperature for 5 min. The solution was quickly cooled to 5° and centrifuged at 48,200 x g for 20 min. The heated supernatant fluid contained 94% of the original activity. The enzyme was kept frozen overnight with no loss in activity.

Protamine Sulfate Fractionation—A volume of 0.10 ml of a 1% protamine sulfate solution was added per ml of heated supernatant fluid. After mixing and centrifuging, the protamine sulfate supernatant fluid was obtained. It contained 86% of the original activity.

Ammonium Sulfate Fractionation—The protamine sulfate supernatant fluid was brought to 40% saturation with solid ammonium sulfate and then centrifuged for 10 min at 48,200 x g. The resulting supernatant fluid was brought to 60% saturation with solid ammonium sulfate. After centrifugation, the precipitate obtained was resuspended in a minimal volume of 0.05 M Tris-HCl (pH 8.0) buffer and dialyzed overnight in 1000 ml of the same buffer. The dialyzed enzyme contained 68% of the original activity.

**RESULTS**

**Activity of Enzyme under Varying Growth Conditions**

Citrate synthase has the highest specific activity of any enzyme studied in *R. capsulata*.

The specific activity of citrate synthase varies greatly from carbon source to carbon source (Table I). The highest specific activity for this enzyme is obtained in acetate-bicarbonate and acetate-grown cells. The specific activities of this enzyme from glucose, dark-grown and light-grown cells, and from pyruvate, dark-grown cells are similar, and they are about one-half the specific activity of the enzyme from acetate-grown cells. The specific activity of this enzyme in pyruvate, light-grown cells is one-half of the value obtained for pyruvate, dark-grown cells. With malate as carbon source, the activity of this enzyme in dark-grown cells is 2-fold greater than the specific activity of this enzyme in the light-grown cells. Malate, light-grown cells, and hydrogen-carbon dioxide, light-grown cells have the lowest citrate synthase specific activity.

**Purification of Citrate Synthase**

**Preparation of Cell-free Extract—Acetate-grown dark-grown cells, 10 g, were resuspended in 100 ml of 0.05 M Tris-HCl (pH 8.0) buffer and exposed to sonic oscillation with a Biosonik III in two 50-ml fractions for a total of 5 min with intermittent cooling in order to keep the temperature below 15°. Unbroken cells and cell debris were removed by centrifugation at 48,200 x g for 30 min in a Sorvall centrifuge. Subsequent steps were carried out at 4°, unless otherwise specified.**

Heat Treatment—The enzyme is relatively heat stable, and can be heated to 60° for 5 min with very little loss in activity. In the presence of AMP, a deinhibitor of the enzyme, it can be heated to 60° with no loss in activity. The Sorvall supernatant fluid was made 0.01 M Tris-HCl with respect to AMP, transferred to a 500-ml Erlenmeyer flask, and heated to 56° in a 60° water bath (12 liters). After reaching 56° (about 5 min), the solution was kept at this temperature for 5 min. The solution was quickly cooled to 5° and centrifuged at 48,200 x g for 20 min. The heated supernatant fluid contained 94% of the original activity. The enzyme was kept frozen overnight with no loss in activity.

Protamine Sulfate Fractionation—A volume of 0.10 ml of a 1% protamine sulfate solution was added per ml of heated supernatant fluid. After mixing and centrifuging, the protamine sulfate supernatant fluid was obtained. It contained 86% of the original activity.

Ammonium Sulfate Fractionation—The protamine sulfate supernatant fluid was brought to 40% saturation with solid ammonium sulfate and then centrifuged for 10 min at 48,200 x g. The resulting supernatant fluid was brought to 60% saturation with solid ammonium sulfate. After centrifugation, the precipitate obtained was resuspended in a minimal volume of 0.05 M Tris-HCl (pH 8.0) buffer and dialyzed overnight in 1000 ml of the same buffer. The dialyzed enzyme contained 68% of the original activity.

DEAE-Cellulose Chromatography—A column, 1 x 30 cm, was packed with DEAE-cellulose (DE 52) to a height of 13 cm. The column was washed with 2 resin bed volumes of 0.05 M Tris-HCl (pH 8.0) buffer. The dialyzed ammonium sulfate fraction was adsorbed onto the column. The column was washed with 1 resin bed volume of buffer before starting the gradient which contained 250 ml of 0.05 M Tris-HCl (pH 8.0) buffer in the mixing chamber and 250 ml of the same buffer containing 0.5 M KCl in the reservoir chamber. Protein was monitored by its absorbance at 290 mJ. The peak activity fractions were pooled, concentrated by precipitation with solid ammonium sulfate, and dialyzed overnight in 0.05 M Tris-HCl buffer (pH 8.0).
Inhibition of dehydroxylation of citrate synthase from *R. capsulata* grown under different growth conditions

Assay conditions were the same as described under "Experimental Procedure," except that DPNH or DPNH plus AMP were added as indicated in the table. The enzyme extracts were prepared by suspending 0.6 g of cells, wet weight, in 12 ml of 0.05 M glycylglycine (pH 7.0) and exposing them to sonic oscillation with a Biosonik III probe for 3 min with cooling. The sonic extracts were centrifuged at 104,000 × g for 1 hour in a Spinco model L ultracentrifuge, and the resulting supernatant fluids were used as the enzyme source. Activity is in micromoles of HS-CoA formed per mg of protein-min.

### Table I

<table>
<thead>
<tr>
<th>Assay conditions</th>
<th>Activity</th>
<th>Percentage of activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>+DPNH (0.1 ml)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>%</td>
</tr>
<tr>
<td>Glucose, dark</td>
<td>0.702</td>
<td>85</td>
</tr>
<tr>
<td>Glucose, light</td>
<td>0.714</td>
<td>20</td>
</tr>
<tr>
<td>Malate, dark</td>
<td>0.467</td>
<td>31</td>
</tr>
<tr>
<td>Malate, light</td>
<td>0.192</td>
<td>20</td>
</tr>
<tr>
<td>Pyruvate, dark</td>
<td>0.711</td>
<td>114</td>
</tr>
<tr>
<td>Pyruvate, light</td>
<td>0.454</td>
<td>21</td>
</tr>
<tr>
<td>Acetate + HCO_3^-</td>
<td>1.80</td>
<td>90</td>
</tr>
<tr>
<td>Acetate, dark</td>
<td>0.039</td>
<td>21</td>
</tr>
<tr>
<td>HI + CO_2, light</td>
<td>0.259</td>
<td>31</td>
</tr>
</tbody>
</table>

* This particular extract had a highly active DPNH oxidase activity.

### Table II

Summary of purification procedure for citrate synthase from *R. capsulata*

One unit equals 1 μmole of HS-CoA formed per min under the conditions described under "Experimental Procedure" (i.e. at 0.18 mM acetyl-CoA).

<table>
<thead>
<tr>
<th>Step</th>
<th>Volume</th>
<th>Protein</th>
<th>Total</th>
<th>Recovery</th>
<th>Specific activity</th>
<th>Fold</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ml</td>
<td>mg/ml</td>
<td>units</td>
<td>%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1. Sorvall supernatant fluid</td>
<td>35</td>
<td>12.6</td>
<td>562</td>
<td>100</td>
<td>1.34</td>
<td></td>
</tr>
<tr>
<td>2. Heated supernatant fluid</td>
<td>35</td>
<td>8.5</td>
<td>525</td>
<td>93.5</td>
<td>1.71</td>
<td>1.3</td>
</tr>
<tr>
<td>3. Protamine sulfate supernatant fluid</td>
<td>36</td>
<td>3.64</td>
<td>485</td>
<td>86.3</td>
<td>3.65</td>
<td>2.7</td>
</tr>
<tr>
<td>4. Ammonium sulfate precipitate</td>
<td>3.1</td>
<td>18.45</td>
<td>379</td>
<td>67.5</td>
<td>6.65</td>
<td>5.0</td>
</tr>
<tr>
<td>5. DEAE-Cellulose column</td>
<td>3.9</td>
<td>2.71</td>
<td>263</td>
<td>46.8</td>
<td>24.9</td>
<td>18.6</td>
</tr>
<tr>
<td>6. DEAE-Sepharose column</td>
<td>2.0</td>
<td>1.50</td>
<td>66</td>
<td>15.1</td>
<td>28.5^a</td>
<td>21.1</td>
</tr>
</tbody>
</table>

* At saturating concentrations of acetyl-CoA (2.2 mM) the specific activity of the enzyme was 45 μmoles of HS-CoA formed per mg of protein-min.

### Characterization of Product and Stoichiometry of Reaction Catalyzed by Citrate Synthase

A reaction mixture containing acetyl-¹⁴C-CoA and nonradioactive oxaloacetate was incubated with 21-fold purified enzyme for 5 min, and the reaction was stopped by heating. A reaction mixture containing no oxaloacetate was run as a control. The reaction mixtures were spotted on silica gel-coated paper strips and developed in Solvent System A. The strips were monitored for radioactivity and sprayed with bromphenol blue (0.3%)-
methyl red (0.1%) (in 95% ethanol) solution to detect organic acids. Unreacted acetyl-CoA remains at the origin, and acetate travels with the solvent front in this system. A new radioactive spot appeared in the strip from the complete reaction mixture and was absent in the strip from the reaction mixture containing no oxaloacetate. The relative mobility of this spot was similar to the relative mobility of citrate.

The definite characterization of citrate as the actual product formed in the presence of purified citrate synthase was carried out enzymatically. Two reaction mixtures were prepared, each containing 100 μmoles of Tris-HCl (pH 8.0), 0.2 μmole of oxaloacetate, and 0.178 μmole of acetyl-CoA in a final volume of 1 ml. To Reaction Mixture A, purified citrate synthase (0.9 μg) was added, to assay for dihydrogenase (Boehringer, 9.8 units per ml; free of DPNH oxidase activity and free of isocitrate dehydrogenase activity) was added, to assay for citrate formed, and the reaction followed. Reaction Mixture B
shown no further decrease in absorbance at 340 nm. The complete Reaction Mixture A showed a further decrease in absorbance at 340 nm caused by the citrate present being cleaved to oxalacetate (and acetate), and the oxalacetate in turn being reduced by DPNH to malate. From the decrease in absorbance, it was calculated that 0.087 μmole of citrate had been formed per original (1 ml) Reaction Mixture A.

Another aliquot, 0.3 ml, of the heated reaction mixtures was withdrawn and added to a 1 ml cuvette containing 75 μmoles of Tris-HCl (pH 8.0) and 0.16 μmole of DTNB in a total volume of 1 ml to assay for the HS-CoA formed. It was calculated that in A, 0.081 μmole of HS-CoA had been formed per original (1 ml) reaction mixture, while in B, 0.005 μmole of HS-CoA had been formed nonenzymatically.

It can thus be concluded that 1 μmole of oxalacetate reacts with 1 μmole of acetyl-CoA to yield 1 μmole of citrate and 1 μmole of HS-CoA.

**Inhibition and Deinhibition of Citrate Synthase**

DPNH inhibits citrate synthase at relatively low concentrations and this inhibition is completely relieved by 5’AMP (Table I). A variety of compounds were tested for their effect on the activity of citrate synthase. At a concentration of 1 mM the following compounds neither activate nor inhibit: fructose-6-P, fructose-1,6-P, 2-keto-3-deoxy-6-phosphogluconate, pyruvate, TPNH, ADP, and ATP. The following compounds, at concentrations of 0.5 and 5 μM, neither activate nor inhibit and do not relieve the inhibition by DPNH: α-keto-glutarate, t-glutamate, fumarate, t-aspartate, t-malate, succinate, and cis-aconitate. At a concentration of 1 mM, dl-isocitrate gives a 1.3-fold activation, but does not relieve the inhibition by DPNH. KCl at a concentration of 0.1 mM gives a 1.3- to 1.4-fold activation (at 0.18 mM acetyl-CoA and 1 mM oxalacetate), and at this concentration also relieves the inhibition by 0.1 mM DPNH.

**Inhibition and Deinhibition of Enzyme under Varying Growth Conditions**—Table I shows the inhibition by DPNH and the deinhibition by 5’AMP of citrate synthase activity in 104,000 x g supernatant fluids obtained from sonic extracts of cells grown under different growth conditions. Although the specific activity of this enzyme varies depending on the conditions of growth, the activity in all cases is inhibited by DPNH, and 5’AMP completely relieves this inhibition.

**Specificity of Deinhibition**—Table III shows the effectiveness of adenylates and of pyridine nucleotides as deinhibitors of DPNH inhibition of the 21-fold purified enzyme. At a concentration of about 1 mM, both AMP and ADP completely relieve the inhibition by 0.1 mM DPNH, while ATP and DPN+ only partially relieve this inhibition; however, at a concentration of about 0.05 mM, AMP completely relieves the inhibition by 0.1 mM DPNH, while ADP only partially relieves this inhibition, and ATP does not relieve it at all. When the concentration of DPNH is increased to 1 mM, 0.5 mM AMP completely relieves this inhibition, 0.5 mM ADP only partially relieves it, and 0.5 mM ATP has no effect. At the same concentration of DPNH, 0.05 mM AMP only partially relieves this inhibition and 0.05 mM ADP or 0.05 mM ATP have no effect.

Other nucleoside monophosphates and analogues of 5’AMP were also tested as deinhibitors. Table IV shows the results obtained. Of those tested only 2’-AMP relieves the inhibition by DPNH. At a concentration of 0.5 mM, both 2’-AMP and 5’-AMP completely relieve the inhibition by 0.1 mM DPNH; however, at a concentration of 0.05 mM, 5’-AMP completely relieves the inhibition by 0.1 mM DPNH, while 2’-AMP only partially relieves this inhibition. When the concentration of DPNH is increased to 0.5 mM, and the nucleoside monophosphate concentration is kept at 0.5 mM, 5’-AMP completely relieves this inhibition while 2’-AMP only partially relieves it.

**Kinetic Parameters**

All the kinetic studies were carried out within the range of enzyme linearity.

**Effect of Acetyl-CoA Concentration**—Fig. 2 shows the dependence of citrate synthase activity on the concentration of acetyl-CoA in the presence and absence of the enzyme’s effector mol-
**TABLE IV**

Nucleosides monophosphates and other analogues as deinhibitors of citrate synthase from *R. capsulata*

Assay conditions were the same as described under "Experimental Procedure," except that the effector molecules were added as indicated in the table. Citrate synthase (specific activity, 45), 0.06 to 0.15 μg was added. The value of 100% activity corresponds to the velocity observed for the reaction mixture containing no effector molecules.

<table>
<thead>
<tr>
<th>Compound</th>
<th>No addition</th>
<th></th>
<th>+0.1 mM DPNH</th>
<th></th>
<th>+0.5 mM DPNH</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Deinhibitor concentration</td>
<td>Percentage of activity</td>
<td>Deinhibitor concentration</td>
<td>Percentage of activity</td>
<td></td>
</tr>
<tr>
<td>No addition</td>
<td>0.46</td>
<td>110</td>
<td>0.46</td>
<td>90</td>
<td></td>
</tr>
<tr>
<td>5'-AMP</td>
<td>0.46</td>
<td>110</td>
<td>0.46</td>
<td>90</td>
<td></td>
</tr>
<tr>
<td>Adenine</td>
<td>0.44</td>
<td>44</td>
<td>0.44</td>
<td>44</td>
<td></td>
</tr>
<tr>
<td>2'-AMP</td>
<td>0.48</td>
<td>48</td>
<td>0.48</td>
<td>48</td>
<td></td>
</tr>
<tr>
<td>3'-AMP</td>
<td>0.51</td>
<td>51</td>
<td>0.51</td>
<td>51</td>
<td></td>
</tr>
<tr>
<td>5'-GMP</td>
<td>0.51</td>
<td>51</td>
<td>0.51</td>
<td>51</td>
<td></td>
</tr>
<tr>
<td>5'-UMP</td>
<td>0.52</td>
<td>52</td>
<td>0.52</td>
<td>52</td>
<td></td>
</tr>
<tr>
<td>5'-TMP</td>
<td>0.45</td>
<td>45</td>
<td>0.45</td>
<td>45</td>
<td></td>
</tr>
<tr>
<td>Cyclic 3',5'-AMP</td>
<td>0.45</td>
<td>45</td>
<td>0.45</td>
<td>45</td>
<td></td>
</tr>
</tbody>
</table>

**Fig. 2.** Dependence of citrate synthase activity on the concentration of acetyl-CoA in the presence and absence of the enzyme's effector molecules. The conditions of the assay were those given under "Experimental Procedure," except that the concentration of acetyl-CoA was varied and either DPNH or AMP or both were added as indicated in the figure; 0.06 to 0.15 μg of citrate synthase (specific activity, 45) was added. The inset is a plot of log v/v/Vmax vs. v against the log of acetyl-CoA (Ac-CoA) concentration. Vmax was taken as 45 μmoles of HS-CoA formed per mg of protein-min for all four cases.

**Fig. 3.** Dependence of citrate synthase activity on the concentration of oxaloacetate in the presence and absence of the enzyme's effector molecules. The conditions of the assay were those given under "Experimental Procedure," except that the concentration of oxaloacetate was varied and DPNH or DPNH plus AMP were added as indicated in the figure; 0.06 to 0.15 μg of citrate synthase (specific activity, 45) was added. The inset shows the corresponding S/v versus S plots. B is a plot of log v/Vmax - v against the log of oxaloacetate (OAA) concentration. Vmax is in micromoles of HS-CoA per mg of protein-min.

**Effect of Oxaloacetate Concentration**—Fig. 3A shows the dependence of citrate synthase activity on the concentration of oxaloacetate at 0.18 mM acetyl-CoA, in the presence and absence of the enzyme's effector molecules. The inset shows the S/v versus S plots. Fig. 3B shows the corresponding Hill plots. The activity at saturating acetyl-CoA concentration, 2.26 mM, is 1.6-fold greater than at 0.18 mM, at which concentration all other work was performed.

**Effect of Acetyl-CoA Concentration**—Fig. 3A shows the dependence of citrate synthase activity on the concentration of acetyl-CoA from 0.99 to 1.47. AMP plus DPNH, or AMP alone, yield the same v value for acetyl-CoA as the control in which no effector molecules are included. AMP thus acts strictly as a deinhibitor of the DPNH inhibition, since it reverses the effect of DPNH on the Smax and v values for acetyl-CoA to the values observed in the absence of the effector molecules.

The activity at saturating acetyl-CoA concentration, 2.26 mM, is 1.6-fold greater than at 0.18 mM, at which concentration all other work was performed.

**Effect of Oxaloacetate Concentration**—Fig. 3A shows the dependence of citrate synthase activity on the concentration of oxaloacetate at 0.18 mM acetyl-CoA, in the presence and absence of the enzyme's effector molecules. The inset shows the S/v versus S plots. Fig. 3B shows the corresponding Hill plots.

DPNH at a concentration of 0.1 mM decreases the Vmax by 70%, increases the S0.5 value for oxaloacetate from 0.021 to 0.055 mM, and does not significantly change the v value for oxaloacetate. AMP, 0.46 mM, in the presence of 0.1 mM DPNH, restores Vmax to the control value obtained in the absence of effector mole-
cules, and decreases the $S_0.5$ for oxalacetate from 0.021 to 0.009 mm. AMP (0.46 mM) alone (not shown in Fig. 3) yields no change in $V_{\text{max}}$, and decreases the $S_0.5$ for oxalacetate from 0.021 to 0.005 mEq. Thus, AMP not only acts as a deinhibitor of the inhibition by DPNH with respect to oxalacetate but also increases the apparent affinity of the enzyme for oxalacetate.

Effect of DPNH Concentration—Fig. 4 shows the effect of DPNH concentration on the citrate synthase activity at two concentrations of acetyl-CoA and in the presence and absence of the deinhibitor AMP. At both 0.115 and at 0.344 mM acetyl-CoA concentrations, in the presence of 0.46 mM AMP, no inhibition is observed even at DPNH concentrations as high as 2.15 mM. In the absence of AMP, at 0.115 mM acetyl-CoA the concentration of DPNH required for one-half maximal inhibition ($I_{0.5}$) is 0.026 mM. When the acetyl-CoA concentration is increased to 0.344 mM, the $I_{0.5}$ for DPNH is increased to 0.074 mM. The inset shows the Hill plots obtained; at both acetyl-CoA concentrations the $n$ value for DPNH is 1.2.

Fig. 5 shows the effect of varying the DPNH:DPNH ratio at two different total diphosphopyridine nucleotide concentrations on the citrate synthase activity. At a total diphosphopyridine nucleotide concentration of 0.2 mM, 50% inhibition occurs when the percentage of reduced diphosphopyridine nucleotide is 28%;
The specific activity of the enzyme was 45 amoles of HS-CoA formed per mg of protein-min.

### Table V

**Kinetic parameters of citrate synthase from *R. capsulata***

<table>
<thead>
<tr>
<th>Compound</th>
<th>Conditions</th>
<th>$S_	ext{0.5}^a$</th>
<th>$A_	ext{0.5}^b$</th>
<th>$I_	ext{0.5}^c$</th>
<th>$S_	ext{0.9}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetyl-CoA</td>
<td>-AMP, -DPNH</td>
<td>0.074</td>
<td>0.93</td>
<td>0.35</td>
<td>0.19</td>
</tr>
<tr>
<td></td>
<td>+AMP, 0.46 mM</td>
<td>0.036</td>
<td>0.83</td>
<td>0.26</td>
<td>0.14</td>
</tr>
<tr>
<td></td>
<td>+DPNH, 0.1 mM</td>
<td>0.024</td>
<td>1.02</td>
<td>0.41</td>
<td>0.12</td>
</tr>
<tr>
<td></td>
<td>AMP, 0.46 mM</td>
<td>0.005</td>
<td>0.82</td>
<td>0.32</td>
<td>0.06</td>
</tr>
<tr>
<td></td>
<td>+DPNH, 0.1 mM</td>
<td>0.028</td>
<td>1.00</td>
<td>0.44</td>
<td>0.14</td>
</tr>
<tr>
<td>Oxalacetate*</td>
<td>-AMP, -DPNH</td>
<td>0.19</td>
<td>1.09</td>
<td>0.49</td>
<td>0.20</td>
</tr>
<tr>
<td></td>
<td>+AMP, 0.46 mM</td>
<td>0.003</td>
<td>0.83</td>
<td>0.38</td>
<td>0.13</td>
</tr>
<tr>
<td></td>
<td>+DPNH, 0.1 mM</td>
<td>0.028</td>
<td>1.00</td>
<td>0.44</td>
<td>0.14</td>
</tr>
<tr>
<td></td>
<td>AMP, 0.46 mM</td>
<td>0.005</td>
<td>0.82</td>
<td>0.32</td>
<td>0.06</td>
</tr>
<tr>
<td></td>
<td>+DPNH, 0.1 mM</td>
<td>0.028</td>
<td>1.00</td>
<td>0.44</td>
<td>0.14</td>
</tr>
<tr>
<td>DPNH</td>
<td>+0.115 mM acetyl-CoA</td>
<td>0.026</td>
<td>1.22</td>
<td>0.36</td>
<td>0.18</td>
</tr>
<tr>
<td></td>
<td>+0.344 mM acetyl-CoA</td>
<td>0.005</td>
<td>0.83</td>
<td>0.32</td>
<td>0.06</td>
</tr>
<tr>
<td>AMP*</td>
<td>+DPNH, 0.10 mM</td>
<td>4.27</td>
<td>1.52</td>
<td>1.13</td>
<td>0.47</td>
</tr>
<tr>
<td></td>
<td>+DPNH, 0.40 mM</td>
<td>20.4</td>
<td>1.77</td>
<td>1.02</td>
<td>0.40</td>
</tr>
<tr>
<td></td>
<td>+DPNH, 0.98 mM</td>
<td>50.8</td>
<td>1.91</td>
<td>1.47</td>
<td>0.83</td>
</tr>
</tbody>
</table>

* $S_	ext{0.5}$ values were obtained from $S/v$ versus $S$ plots (Fig. 3A).
* Values obtained from Hill plots (Figs. 2, 3B, 4, and 6B).
* At 0.15 mM acetyl-CoA.

while at a total diphosphopyridine nucleotide concentration of 1 mM, 50% inhibition occurs when the percentage of reduced diphosphopyridine nucleotide is 17.5%. The presence of low levels of AMP (20 μM) at a total diphosphopyridine nucleotide concentration of 1 mM increases the percentage of reduced diphosphopyridine nucleotide required to yield 50% inhibition from 17.5 to 54%.

**Effect of 5'-AMP Concentration**—Fig. 6A shows the effect of 5'-AMP concentration, in the presence of three different levels of DPNH, on the citrate synthase activity. Increasing amounts of DPNH increase the degree of sigmoidicity of the deinhibitor saturation curve. At low 5'-AMP concentrations, the higher the DPNH concentration the greater the degree of inhibition; however, increasing the 5'-AMP concentration completely overcomes the inhibition by DPNH. The $1/\Delta_l$ versus $1/S$ plot shown in the inset is nonlinear. Fig. 6B shows the corresponding Hill plots. The concentration of 5'-AMP required for one-half maximal activation (deinhibition) is $4.27 \text{ μM}$ in the presence of 0.1 mM DPNH, 26.4 μM in the presence of 0.49 mM DPNH, and 50.8 μM in the presence of 0.98 mM DPNH. The $n$ value for 5'-AMP is 1.52 in the presence of 0.1 mM DPNH, 1.77 in the presence of 0.49 mM DPNH, and 1.91 in the presence of 0.98 mM DPNH.

The kinetic parameters of citrate synthase from *R. capsulata* are summarized in Table V.

### Table VI

**Inhibition and deinhibition of citrate synthase from *R. rubrum* grown under different growth conditions**

Assay conditions were the same as described under "Experimental Procedure," except that DPNH or DPNH plus AMP were added as indicated in the table. The enzyme extracts were prepared by suspending 0.6 g of cells, wet weight, in 12 ml of 0.05 M glycylglycine (pH 7.0) and exposing them to sonic oscillation with a Biosonik III probe for 3 min with cooling. The sonic extracts were centrifuged at 104,000 × g for 1 hour in a Spinco model L ultracentrifuge, and the resulting supernatant fluids were used as the enzyme source. Activity is in micromoles of HS-CoA formed per mg of protein-min.

<table>
<thead>
<tr>
<th>Growth conditions</th>
<th>Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No effect added</td>
</tr>
<tr>
<td>Malate, light</td>
<td>0.153</td>
</tr>
<tr>
<td>Malate, dark</td>
<td>0.423</td>
</tr>
<tr>
<td>$\text{H}_2 + \text{CO}_2$, light</td>
<td>0.206</td>
</tr>
</tbody>
</table>

### Discussion

The specific activity of citrate synthase was found to be dependent on the nature of the carbon source and was 2-fold greater in cells grown aerobically in the dark than in cells grown semiaerobically in the light, when either malate or pyruvate were the carbon sources (Table I). With *R. rubrum* grown on malate as carbon source, the specific activity in the dark-grown cells was about 3-fold greater than the specific activity in the light-grown cells (Table VI). Anderson and Fuller (15) obtained similar results with citrate synthase from *R. rubrum* grown on
malate in a minimal salts medium. These observations would be consistent with a more active tricarboxylic acid cycle in the dark than in the light. Since an important function of the tricarboxylic acid cycle is to produce reducing power, the activity of this cycle would be expected to be decreased in the light where reducing power could also be obtained from photosynthesis in a photosynthetic bacterium like R. capsulata. This regulation could be accomplished by controlling the amount of enzyme synthesized, or by regulating the activity of the enzyme synthesized, or both. Although the amount of citrate synthase in the light was lower than in the dark (on malate or pyruvate), it still showed the highest activity of any of the enzymes investigated in this organism. In light of this, the allosteric regulation of the activity of this enzyme becomes of great interest.

Citrate synthase in R. capsulata catalyzes a physiologically irreversible reaction and it can be considered as the first enzyme in the tricarboxylic acid cycle. Citrate synthase in this organism was found to be a regulatory enzyme. DPNH was a strong inhibitor of this enzyme and AMP was found to be the best deinhibitor.

Although the levels of DPNH and DPN$^+$ in R. capsulata are not known, the levels of these cofactors have been measured in Escherichia coli (16); the concentration of DPNH in glucose-grown cells was 2.05 mM and in succinate-grown cells it was 1.2 mM, and the concentration of DPN$^+$ was 1.08 and 1.44 mM in glucose- and in succinate-grown cells, respectively. Jackson and Crofts (17) have shown that in R. rubrum under dark aerobic conditions, the total diphosphopyridine nucleotides were present as DPN$^+$; while under light anaerobic conditions, 70% of the total diphosphopyridine nucleotide pool was in the form of DPNH and 30% in the form of DPN$^+$.

Under conditions of “low energy charge” (i.e. low ATP:ADP + AMP ratio) (18) and “low reducing power” (i.e. low DPNH:DPN$^+$ ratio) the activity of citrate synthase would be maximal resulting in an active tricarboxylic acid cycle. The increase in “reducing power” in the cell, caused by substrate oxidation in the dark or by photosynthesis in the light, with the concomitant increase in energy charge caused by an “excess of ATP production” over that utilized by the cell, would result in the inhibition of citrate synthase of R. capsulata. This inhibition would decrease the activity of the tricarboxylic acid cycle.

Jangaard et al. (3) found ATP to be an inhibitor of citrate synthase from bovine liver, bovine heart, and E. coli. Weitzman (4) found that the enzyme from E. coli was very sensitive to DPNH inhibition. α-Ketoglutarate has been reported to also be an inhibitor of the enzyme from E. coli (19). Neither ATP nor α-ketoglutarate inhibited the enzyme from R. capsulata under the conditions of the assay used. It should be pointed out that Jangaard et al. (3) showed that ATP inhibition of the E. coli enzyme was dependent on pH. Although a similar study was not made in the case of the enzyme from R. capsulata, the pH of the reaction mixtures in the present study (8.0) was the pH reported where the E. coli enzyme was most sensitive to ATP inhibition.

The finding of DPNH inhibition and AMP deinhibition of citrate synthase in R. capsulata is consistent with the observations of Weitzman and Jones (2). They reported that the enzyme from gram-negative organisms was inhibited by DPNH. In this group, those organisms (strict aerobes) that do not metabolize glucose via the Embden-Meyerhof pathway but utilize an alternate pathway (e.g. Entner-Doudoroff) were very sensitive to deinhibition by AMP, while those organisms (facultative anaerobes) that do metabolize glucose via the Embden-Meyerhof pathway were less sensitive to deinhibition by AMP. Organisms included in the former group like Pseudomonas sp. (20) and Xanthomonas sp. (21) metabolize glucose via the Entner-Doudoroff pathway (22). R. capsulata, which also metabolizes glucose via the Entner-Doudoroff pathway but is not a strict aerobe, would now be included in this group. E. coli was included in the latter group. The inclusion of E. coli citrate synthase in the AMP-insensitive group would appear to be inconsistent with Weitzman’s (5) previous report that the inhibition by DPNH (1 mM) in E. coli is “virtually completely overcome” by ATP (0.3 mM) under very similar conditions to those utilized in his more recent investigation (2) (i.e. DPNH, 0.8 mM; AMP, 0.5 mM). This discrepancy reported for E. coli should eventually be resolved when more complete kinetic analysis of the inhibition-deinhibition relationship is undertaken for that enzyme.

Weitzman and Jones (2) concluded that organisms in which AMP (or ADP), acting as a “low energy signal,” activates the key glycolytic enzymes such as phosphofructokinase or pyruvate kinase, would not require a similar low energy signal at the level of their citrate synthase. On the other hand, organisms in which the Embden-Meyerhof pathway is absent or in which there is no regulation of its key enzymes would require a low energy signal to control the tricarboxylic acid cycle at the level of the entry to the cycle, namely citrate synthase. The latter appears to be the case with R. capsulata.

Weitzman and Dunmore (23) found that α-ketoglutarate (1 mM) only inhibited the citrate synthase of the Enterobacteriaceae. Wright, Maeba, and Sanwal (19) have shown that α-ketoglutarate (a tricarboxylic acid cycle intermediate which is not only used for energy generation but also for biosynthesis of glutamate) acted as a feedback inhibitor of citrate synthase from E. coli. The citrate synthase from R. capsulata was not inhibited by α-ketoglutarate. Citrate synthase activity is not only necessary for energy production but is also necessary for biosynthesis of the glutamate family of amino acids. In light of this, it is interesting to note that the enzyme from R. capsulata could not be completely inhibited by DPNH, a high energy signal (Fig. 4). The residual (noninhibitable) activity could be considered to always be active for biosynthetic purposes.

Rindt and Ohmann (24) have recently reported that in Rhodopseudomonas spheroides (an organism closely related to R. capsulata), ribulose-5-P kinase, a key enzyme of the reductive pentose-phosphate cycle, is activated by DPNH (the inhibitor of citrate synthase) and is inhibited by AMP (the deinhibitor of this enzyme). The fructose-1,6-diphosphate (1,6-P$_2$)-inhibitor of citrate synthase. This effector relationship is consistent with the findings reported here for R. capsulata. Under conditions of active growth in the light, the DPNH levels would be expected to increase while the AMP levels would be expected to decrease. This would lead to activation of ribulose-5-P kinase and inhibition of citrate synthase resulting in low tri-carboxylic acid cycle activity and high reductive pentose-phosphate cycle activity. The reciprocal relationship between citrate synthase and ribulose-5-P kinase in photosynthetic bacteria with respect to their modulation by the energy charge and the “reducing state” of the cell could be responsible for the regulation and interaction between two of the carbon pathways present in these organisms.
REFERENCES
Citrate Synthase: A REGULATORY ENZYME FROM RHODOPSEUDOMONAS CAPSULATA
Leon Eidels and Jack Preiss


Access the most updated version of this article at http://www.jbc.org/content/245/11/2937

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/245/11/2937.full.html#ref-list-1