Succinic ThioKInase

II. KINETIC STUDIES: INITIAL VELOCITY, PRODUCT INHIBITION, AND EFFECT OF ARSENATE*

Sungman Cha†‡ and R. E. Parks, Jr.‡

From the Department of Pharmacology and Toxicology, University of Wisconsin Medical School, Madison 6, Wisconsin

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The proposed reaction mechanisms for succinic thioKInases from various sources are well summarized in recent reviews by Jaenicke and Lynen (2) and by Hager (3). On the basis of various isotope exchange studies with the succinic thioKInase from spinach, Kaufman (4) proposed a reaction sequence that includes phosphoryl coenzyme A as an enzyme-bound intermediate. This mechanism was supported by Mazur and Rodwell (5) on the basis of 32P-orthophosphate-inosine triphosphate exchange studies with the enzyme purified from pig kidney cortex. The reported resolution of Escherichia coli succinic thioKInase into two fractions, CoA phosphokinase and phosphoryl-succinic thiokinase, and the identification of phosphoryl coenzyme A as an enzyme-bound intermediate. This mechanism was supported by Mazur and Rodwell (5) on the basis of 32P-orthophosphate-inosine triphosphate exchange studies with the enzyme purified from pig kidney cortex. The reported resolution of Escherichia coli succinic thioKInase into two fractions, CoA phosphokinase and phosphoryl-succinic thiokinase, and the identification of phosphoryl coenzyme A as a free intermediate (6) are still open to question (2, 3, 5, 7).

Succinic thioKInase preparations from pig heart (8), pig kidney cortex (5), and spinach leaves (4) have been reported to catalyze the arsenolysis of succinyl-CoA in the presence of magnesium ion. Also, arsenolysis of inosine triphosphate was catalyzed by a relatively crude preparation of succinic thioKInase, but not by a purer enzyme preparation from pig kidney cortex (5). Kaufman proposed the formation of unstable arsenoyl-CoA and subsequent hydrolysis as the mechanism of arsenolysis catalyzed by succinic thioKInase from spinach (4).

Analyses of initial velocity and product inhibition data obtained in this laboratory with succinic thioKInase from pig heart led to the postulation of a reaction sequence which is somewhat different from those proposed by other workers. Also presented are the inhibitory effects of arsenate on the succinic thioKInase reaction and those of normal reactants on the arsenolysis of succinyl-CoA.

EXPERIMENTAL PROCEDURE

Materials and Assay Methods—The purification procedure for the enzyme and substrates and the assay methods were described in the previous paper (8). Enzyme preparations of various purities (specific activity, 20 to 230 units per mg) were used, and the concentration of MgCl2 was held constant at 10 mM and 7.4, respectively. AmGDP1 and azaGTP were used

* Aided by Grant T-94E from the American Cancer Society.
† Taken in part from a dissertation submitted to the Graduate School of the University of Wisconsin in partial fulfillment of the requirements for the degree of Doctor of Philosophy, June 1963.
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The abbreviations used are: azaGDP and azaGTP, 8-aza-guanosine di- and triphosphate, respectively.

in some of the initial velocity studies because, in contrast to GDP and GTP, these compounds can be studied conveniently at nonsaturating concentrations, i.e. near their apparent Michaelis constants.

The arsenolysis reaction was followed by recording the absorbance decrease at 235 μM, in a manner similar to that described for the normal reaction. The reaction mixture contained Tris-acetate, pH 7.4, 100 μM, and MgCl2, 10 μM, in addition to the enzyme and various amounts of succinyl-CoA and arsenate (Na2HAsO4, analytical grade). Approximately, 0.1 unit of enzyme was required to obtain a convenient reaction rate (a decrease in absorbance of approximately 0.01 to 0.04 per minute). When the arsenolysis was strongly inhibited, a marked lag period was observed, sometimes lasting as long as 10 minutes at higher concentrations of inhibitor; i.e. the rate of decomposition of succinyl-CoA was slow during this lag period, then gradually increased to a maximum. No adequate explanation for this phenomenon is apparent. The "initial" velocities presented in this paper were measured after the lag periods were over. The effects of arsenate on the rate of the normal reaction were studied by measuring the formation of GDP in a system coupled to pyruvic kinase and lactic dehydrogenase (8).

Analyses of Kinetic Data—A preliminary Lineweaver-Burk plot (1/v versus 1/S) was made for each set of data to see whether the plot appeared to be a straight line. All the data, except those showing obvious curvature on the preliminary plots, were processed by the computer program developed and generously provided by W. W. Cleland (9). This program is designed for the fit of data by the method of least squares directly to a hyperbola, and it provides various kinetic parameters such as the intercepts of the slopes, to give K, values, all with their respective standard errors of estimation. The theoretical considerations of Cleland concerning multireactant enzyme kinetics have been applied to the interpretation of the data, and the nomenclature, such as competitive, uncompetitive, noncompetitive, variable substrate, changing fixed substrate, product inhibition, initial velocity pattern, Ki, K, linear inhibition, etc., have been used as defined by Cleland (10-12). Inhibition constants were estimated from the replots of the intercepts to give K, and from replots of the slopes, to give K.

RESULTS

Initial Velocity Studies—When azaGDP and phosphate were the variable and changing fixed substrates, respectively, a parallel pattern was obtained (Fig. 1). Since the third substrate, succinyl-CoA, was held constant at a nonsaturating concentra...
tion (0.05 mM), the data shown in Fig. 1 indicate that at least one product is released from the enzyme between the time of addition of the two substrates, azaGDP and phosphate, in the reaction sequence. A similar parallel pattern was obtained when phosphate and succinyl-CoA were the variable and changing fixed substrates and the third substrate, azaGDP, was held constant at 0.05 mM (Fig. 2). This indicates that at least one product is released between the addition of the two substrates, succinyl-CoA and phosphate. In contrast to the results of these two experiments, an intersecting pattern was obtained when succinyl-

![Fig. 1. Plot of reciprocal of initial velocity (v) versus reciprocal of concentration of azaGDP (millimolar). The concentrations of phosphate, the changing fixed substrate, were 2.0, 1.0, 0.7, and 0.5 mM. Held constant were succinyl-CoA at 0.05 mM, Tris-acetate buffer, pH 7.4, at 100 mM, and MgCl₂ at 10 mM. Velocities are expressed as the decrease in absorbance at 235 nm per minute. Kinetic parameters (+ standard error) at phosphate concentrations of 2.0, 1.0, 0.7, and 0.5 mM are: apparent $K_m$ values for azaGDP, 0.056 ± 0.009, 0.082 ± 0.012, 0.082 ± 0.010, and 0.0474 ± 0.007 mM; vertical intercepts ($V/V$ $\text{min}^{-1}$), 19.9 ± 2.3, 26.2 ± 2.1, 38.2 ± 4.0, and 47.1 ± 3.7; slopes ($V/V$ $\text{mM}^{-1}$ $\text{min}^{-1}$), 1.91 ± 0.13, 2.16 ± 0.14, 2.00 ± 0.20, and 2.19 ± 0.18; with 6, 6, 6, and 5 degrees of freedom, respectively.](http://www.jbc.org/)

![Fig. 2. Plot of reciprocal of initial velocity (v) versus reciprocal of concentration of phosphate (millimolar). The concentrations of succinyl-CoA, the changing fixed substrate, were 0.02, 0.03, and 0.05 mM. Held constant were Tris-acetate buffer, pH 7.4, at 100 mM, azaGDP at 0.05 mM, and MgCl₂ at 10 mM. Velocities are expressed as the decrease in absorbance at 235 nm per minute. Kinetic parameters (+ standard error) at azaGDP concentrations of 0.02, 0.03, and 0.05 mM are: apparent $K_m$ values for succinyl-CoA, 0.010 ± 0.002, 0.023 ± 0.008, and 0.057 ± 0.012 mM; vertical intercepts ($V/V$ $\text{min}^{-1}$), 48.1 ± 2.4, 44.5 ± 4.4, and 103.6 ± 4.5; slopes ($V/V$ $\text{mM}^{-1}$ $\text{min}^{-1}$), 23.2 ± 2.5, 24.7 ± 4.4, and 18.8 ± 3.7; with 3, 3, and 4 degrees of freedom, respectively.](http://www.jbc.org/)

![Fig. 3. Plot of reciprocal of initial velocity (v) versus reciprocal of concentration of succinyl-CoA (millimolar). The concentrations of azaGDP, the changing fixed substrate, were 0.1, 0.05, and 0.02 mM. Held constant were phosphate at 1.0 mM, Tris-acetate buffer, pH 7.4, at 100 mM, and MgCl₂ at 10 mM. Velocities are expressed as the decrease in absorbance at 235 nm per minute. Kinetic parameters (+ standard error) at azaGDP concentrations of 0.1, 0.05, and 0.02 mM are: apparent $K_m$ values for succinyl-CoA, 0.010 ± 0.032, 0.023 ± 0.0047, and 0.057 ± 0.0035 mM; vertical intercepts ($V/V$ $\text{min}^{-1}$), 44.7 ± 5.7, 49.1 ± 3.7, and 79.7 ± 5.1; slopes ($V/V$ $\text{mM}^{-1}$ $\text{min}^{-1}$), 0.47 ± 0.18, 1.14 ± 0.15, and 2.57 ± 0.41; with 3, 5, and 4 degrees of freedom, respectively.](http://www.jbc.org/)

![Fig. 4. Plot of reciprocal of initial velocity (v) versus reciprocal of concentration of CoA (millimolar). The concentrations of azaGTP, the changing fixed substrate, were 0.1, 0.05, and 0.02 mM. Held constant were Tris-acetate buffer, pH 7.4, at 100 mM, succinyl-CoA at 1.0 mM, and MgCl₂ at 10 mM. Velocities are expressed as the increase in absorbance at 235 nm per minute. Kinetic parameters (+ standard error) at azaGTP concentrations of 0.10, 0.05, and 0.03 mM are: apparent $K_m$ values for CoA, 0.0047 ± 0.00018, 0.0048 ± 0.00009, and 0.0087 ± 0.00053 mM; vertical intercepts ($V/V$ $\text{min}^{-1}$), 31.0 ± 1.1, 49.5 ± 2.4, and 75.6 ± 1.3; slopes ($V/V$ $\text{mM}^{-1}$ $\text{min}^{-1}$), 0.144 ± 0.032, 0.258 ± 0.112, and 0.512 ± 0.068; with 6, 6, and 5 degrees of freedom, respectively.](http://www.jbc.org/)
**FIG. 5** (top left). Plot of reciprocal of initial velocity (v) versus reciprocal of concentration of GTP (millimolar). The concentrations of succinate, the changing fixed substrate, were 50, 10, 1, and 0.4 mM. Held constant were CoA at 0.1 mM, Tris-chloride buffer, pH 7.4, at 100 mM, and MgCl₂ at 10 mM. Velocities are expressed as the increase in absorbance at 235 nm per minute. Kinetic parameters (± standard error) at succinate concentrations of 50, 10, 1, and 0.4 mM are: apparent K_m values for GTP, 0.0137 ± 0.0032, 0.0230 ± 0.0045, 0.0119 ± 0.0014, and 0.0054 ± 0.0016 mM; vertical intercepts (1/v min⁻¹), 30.5 ± 2.0, 31.9 ± 2.3, 55.8 ± 1.7, and 60.6 ± 1.7; slopes (1/v mM⁻¹ min⁻¹), 0.42 ± 0.07, 0.74 ± 0.09, 0.67 ± 0.06, and 0.49 ± 0.12; with 10, 10, 25, and 9 degrees of freedom, respectively.

**FIG. 6** (top right). Plot of reciprocal of initial velocity (v) versus reciprocal of concentration of GDP (millimolar). The concentrations of the inhibitory product, GTP, were 0, 0.05, and 0.10 mM. Held constant were potassium phosphate, pH 7.4, at 1.0 mM, GDP at 0.05 mM, Tris-acetate, pH 7.4, at 100 mM, and MgCl₂ at 10 mM. Velocities are expressed as the decrease in absorbance at 235 nm per minute. Kinetic parameters (± standard error) at CoA concentrations of 0, 0.02, and 0.04 mM are: apparent K_m values for GDP, 0.0011 ± 0.0010, 0.0093 ± 0.0032, and 0.0124 ± 0.0007 mM; vertical intercepts (1/v min⁻¹), 27.6 ± 0.83, 26.8 ± 0.24, and 28.2 ± 0.43; slopes (1/v mM⁻¹ min⁻¹), 0.031 ± 0.026, 0.030 ± 0.020, and 0.034 ± 0.015; with 13, 6, and 8 degrees of freedom, respectively. Inhibition constants of GTP are approximately: K_i = K_i, = 1 X 10⁻³ M.

**FIG. 7** (center left). Plot of reciprocal of initial velocity (v) versus reciprocal of concentration of succinyl-CoA (millimolar). The concentrations of the inhibitory product, CoA, were 0, 0.02, and 0.04 mM. Held constant were potassium phosphate, pH 7.4, at 1.0 mM, GDP at 0.05 mM, Tris-acetate, pH 7.4, at 100 mM, and MgCl₂ at 10 mM. Velocities are expressed as the decrease in absorbance at 235 nm per minute. Kinetic parameters (± standard error) at GTP concentrations of 0, 0.02, and 0.04 mM are: apparent K_m values for succinyl-CoA, 0.014 ± 0.002, 0.020 ± 0.004, and 0.012 ± 0.008 mM; vertical intercepts (1/v min⁻¹), 58.0 ± 3.4, 60.8 ± 4.3; slopes (1/v mM⁻¹ min⁻¹), 0.90 ± 0.12, 2.22 ± 0.24, and 2.81 ± 0.31; with 22, 4, and 4 degrees of freedom, respectively. Inhibition constants of CoA are approximately: K_i = K_i, = 2 X 10⁻⁶ M.

**FIG. 8** (center right). Plot of reciprocal of initial velocity (v) versus reciprocal of concentration of succinate (millimolar). The concentrations of the inhibitory product, succinate, were 0, 0.5, and 2.0 mM. Held constant were phosphate at 1.0 mM, GDP at 0.05 mM, Tris-acetate buffer, pH 7.4, at 100 mM, and MgCl₂ at 10 mM. Velocities are expressed as the decrease in absorbance at 235 nm per minute. Kinetic parameters (± standard error) at succinate concentrations of 0, 0.5, and 2.0 mM are: apparent K_m values for succinyl-CoA, 0.14 ± 0.002, 0.020 ± 0.004, and 0.023 ± 0.008 M; vertical intercepts (1/v min⁻¹), 63.5 ± 1.8, 58.0 ± 3.4, 60.8 ± 4.3; slopes (1/v mM⁻¹ min⁻¹), 0.90 ± 0.12, 2.22 ± 0.24, and 2.81 ± 0.31; with 22, 4, and 4 degrees of freedom, respectively.
FIG. 11 (left). Plot of reciprocal of initial velocity (v) versus reciprocal of concentration of succinate (millimolar). The concentrations of the inhibitory product, phosphate (potassium salt, pH 7.4) were 0, 5, 10, and 20 mM. Succinate concentrations were varied in the range from 0.2 to 50 mM. The concentration of Tris buffer (sum of succinate and chloride) was held constant at approximately 115 mM. The concentration of MgCl₂ at 10 mM. Also held constant were GTP and CoA at 0.1 mM and MgCl₂ at 10 mM. Velocities are expressed as the increase in absorbance per minute at 235 nm. Each point on the figure represents the average values from two assays. No statistical analysis was performed; lines were drawn by inspection.

CoA and azaGDP were the variable and changing fixed substrates, respectively, and phosphate was held constant at 1.0 mM (Fig. 3). This indicates that no product is released from the enzyme between the time of addition of the two substrates, azaGDP and succinyl-CoA.

Similar initial velocity studies were conducted from the reverse direction. As shown in Fig. 4, an intersecting pattern was obtained when CoA was varied at several changing fixed levels of azaGTP and at a constant concentration of succinate (1.0 mM). This indicates that no product is released between the addition of the two substrates, CoA and azaGTP, to the enzyme in the reaction sequence. On the other hand, when GTP and succinate were variable and changing fixed substrates, respectively, and CoA was held constant at 0.1 mM, a mixed pattern was obtained (Fig. 5). In this particular experiment the concentration range of the changing fixed substrate, succinate, was much greater than that employed in the other experiments. The lines corresponding to succinate concentrations of 10 mM or lower are parallel, but the line corresponding to 50 mM succinate intersects the other lines. This may be interpreted as follows: at low concentrations of succinate, a product is released from the enzyme before succinate binds, but when the succinate concentration is sufficiently high, it can bind with the enzyme even before a product is released. This suggested the occurrence of an alternative pathway, which was further supported by the product inhibition studies presented below.

Product Inhibition Studies—When GDP and GTP were the variable substrate and the inhibitory product, respectively, competitive inhibition was observed as shown in Fig. 6. This suggests that GDP and GTP share a common binding site on the enzyme surface. Similarly, competition was observed between succinyl-CoA and CoA (Fig. 7). When succinyl-CoA was the inhibitor, the inhibition constants of phosphate are approximately: $K_i = 7 \times 10^{-2} M$, $K_{is} = \infty$.

FIG. 12 (right). Plot of reciprocal of initial velocity (v) versus reciprocal of concentration of phosphate (millimolar). The concentrations of the inhibitory product, phosphate (potassium salt, pH 7.4) were 0, 2, and 5 mM. Phosphate (potassium salt, pH 7.4) concentrations were varied in the range from 0.2 to 50 mM. Held constant were Tris-acetate buffer, pH 7.4, at 100 mM, MgCl₂ at 10 mM, and both GDP and succinyl-CoA at 0.1 mM. Velocities are expressed as the decrease in absorbance per minute at 235 nm. Each point on the figure represents the average values from two assays. No statistical analysis was performed; lines were drawn by inspection.
**TABLE I**

*Apparent Michaelis constants (mM) for various substrates of succinic thiokinase from pig heart*

**A. Forward reaction**

<table>
<thead>
<tr>
<th>Variable substrate</th>
<th>Concentration of other substrates</th>
<th>Apparent $K_m$ of variable substrate</th>
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</thead>
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<tr>
<td></td>
<td>Succinyl-CoA</td>
<td>GDP</td>
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<td>--------------------</td>
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</tr>
<tr>
<td>Succinyl-CoA</td>
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<td>GDP</td>
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<tr>
<td>AzaGDP</td>
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<td></td>
<td>0.02-0.1</td>
<td>1.0</td>
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<tr>
<td>Phosphate</td>
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<tr>
<td></td>
<td>0.05</td>
<td>0.02-0.10</td>
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</tbody>
</table>

**B. Reverse reaction**

<table>
<thead>
<tr>
<th>Variable substrate</th>
<th>Concentration of other substrates</th>
<th>Apparent $K_m$ of variable substrate</th>
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</thead>
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<td></td>
<td>CoA</td>
<td>GTP</td>
</tr>
<tr>
<td>--------------------</td>
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<td>-----</td>
</tr>
<tr>
<td>CoA</td>
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<td></td>
</tr>
<tr>
<td>GTP</td>
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<td>0.02-0.1</td>
</tr>
<tr>
<td></td>
<td>0.05</td>
<td>0.4-1.0</td>
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<tr>
<td>AzaGTP</td>
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<tr>
<td></td>
<td>0.05</td>
<td></td>
</tr>
<tr>
<td>Succinate</td>
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<td>0.02-0.07</td>
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<tr>
<td></td>
<td>0.1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.05</td>
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</table>

* $K_m$ values estimated from the data obtained with concentrations of variable substrates less than 2 mM.

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**Fig. 13.** Plot of reciprocal of initial velocity (v) versus reciprocal of concentration of arsenate (millimolar). The concentrations of the inhibitor, phosphate, were 0, 0.01, and 0.05 mM. The concentrations of succinyl-CoA at 0.05 mM, Tris-acetate, pH 7.4, at 100 mM, and MgCl₂ at 10 mM. Velocities are expressed as the decrease in absorbance per minute at 235 nm. Kinetic parameters (± standard error) at phosphate concentrations of 0, 0.01, and 0.05 mM are: apparent $K_m$ values of arsenate, 1.39 ± 0.17, 1.72 ± 0.07, and 2.80 ± 0.84 mM; vertical intercepts (1/A min⁻¹), 12.3 ± 0.56, 13.8 ± 0.33, and 14.3 ± 2.90; slopes (1/A mm⁻¹ min⁻¹), 17.1 ± 0.6, 23.7 ± 0.4, and 40.0 ± 4.1; with 12, 3, and 3 degrees of freedom, respectively. Inhibition constants of phosphate are approximately: $K_{in} = \infty$, $K_{is} = 3 \times 10^{-5}$ M.
Fig. 14 (left). Plot of reciprocal of initial velocity \( (v) \) versus reciprocal of concentration of arsenate (millimolar). The concentrations of the inhibitor, GTP, were 0, 0.0025, 0.005, and 0.01 mM. Held constant were succinyl-CoA at 0.05 mM, Tris-acetate buffer, pH 7.4, at 100 mM, and MgCl₂ at 10 mM. Velocities are expressed as the decrease in absorbance per minute at 235 nm. Kinetic parameters (± standard error) at GTP concentrations of 0, 0.0025, 0.005, and 0.01 mM are: apparent \( K_m \) values for arsenate, 0.70 ± 0.12, 0.43 ± 0.10, 0.71 ± 0.21, and 1.31 ± 0.41 mM; vertical intercepts \( (1/A \text{ min}^{-1}) \), 12.6 ± 1.3, 25.4 ± 2.0, 25.5 ± 4.7, and 29.7 ± 6.8; slopes \( (1/A \text{ min}^{-1}) \), 8.8 ± 0.6, 10.9 ± 1.3, 17.8 ± 2.2, and 39.1 ± 3.5; with 8, 3, 3, and 3 degrees of freedom, respectively.

Apparent Michaelis Constants—The apparent \( K_m \) values for various substrates are summarized in Table I. The \( K_m \) values reported by other workers are also listed for the purpose of comparison.

Arsenolysis of Succinyl-CoA—The ability of succinic thiokinase to catalyze the arsenolysis of succinyl-CoA in the presence of Mg²⁺ (13), and the failure to catalyze the arsenolysis of GTP (4), were confirmed with the pig heart enzyme preparation of the highest purity prepared in this laboratory (specific activity, 230 units per mg). The average of apparent \( K_m \) values for arsenate obtained in several experiments (Figs. 13, 14, 16, and 17) was \( 1.0 \times 10^{-3} \text{ M} \); in all these experiments succinyl-CoA and MgCl₂ were kept constant at 0.5 and 10 mM, respectively, and the pH was 7.4. The apparent \( K_m \) for succinyl-CoA (arsenate, 1.0 mM; MgCl₂, 10 mM; pH 7.1) was approximately \( 1 \times 10^{-3} \text{ M} \). In the kinetic studies of the arsenolysis reaction, arsenate was varied at concentrations lower than 2 mM because at higher concentrations the Lineweaver-Burk plots were found to bend downward, as is the case with succinate and phosphate (Figs. 11 and 12).

The inhibition of the arsenolysis by phosphate (13) was reinvestigated. As shown in Fig. 13, phosphate was a potent inhibitor \( (K_i = 3 \times 10^{-3} \text{ M}) \) which competed with arsenate. GTP was an even stronger inhibitor (Fig. 14). The type of inhibition by GTP may be a complicated one, perhaps S-parabolic; i.e., the replot of slope against GTP concentration resembles a parabola (Fig. 15). Whether this seemingly complicated type of inhibition is real or an artifact has not been established. Since the velocities were taken after the lag period, a systematic bias might have been introduced in the determination of reaction rates. The \( K_i \) value estimated under the assumption that the inhibition is of the linear competitive type was approximately \( 5 \times 10^{-4} \text{ M} \). GDP was also competitive with arsenate (Fig. 16), but its inhibitory effect \( (K_i = 4.0 \times 10^{-4} \text{ M}) \) was far less potent than that of phosphate or GTP. Succinate was at best a very weak competitor of arsenate \( (K_i \approx 6.7 \times 10^{-2} \text{ M}) \) (Fig. 17). The
inhibition was so weak that it was not detected in a preliminary study (1).

In Table II the approximate $K_i$ values for phosphate, succinate, GDP, and GTP as inhibitors of the arsenolysis are compared with the apparent $K_m$ values of these compounds as substrates for the normal reaction.

**Inhibitory Effect of Arsenate on Normal Reaction**—When GTP was varied and succinate and CoA were kept constant, arsenate caused noncompetitive inhibition (Fig. 18). On the other hand, when succinate was the variable substrate and both GTP and CoA were held at a constant concentration, arsenate was shown to be an uncompetitive inhibitor (Fig. 19). This is in contrast to either the competitive or the noncompetitive inhibition exhibited by phosphate when succinate is the variable substrate. These different types of inhibition exhibited by phosphate and arsenate suggest that the two compounds react with different enzyme-substrate complexes.

**Discussion**

Because of the limited number of available kinetic parameters and their relatively large variances, no attempt has been made to estimate the true Michaelis constant, i.e. $K_m$ of a substrate at infinite concentrations of the other two substrates. Nevertheless, it is noteworthy that the apparent $K_m$ values are generally much lower than those previously reported by other workers (Table I), and that the apparent $K_m$ of GTP is of the same order

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**Table II**

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Apparent $K_m$</th>
<th>$K_i$, arsenolysis</th>
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<tr>
<td>GDP</td>
<td>$2 \times 10^{-4}$</td>
<td>$4 \times 10^{-4}$</td>
</tr>
<tr>
<td>GTP</td>
<td>$5 \times 10^{-4}$</td>
<td>$5 \times 10^{-4}$</td>
</tr>
<tr>
<td>Phosphate</td>
<td>$1.5 \times 10^{-4}$</td>
<td>$3 \times 10^{-4}$</td>
</tr>
<tr>
<td>Succinate</td>
<td>$4.0 \times 10^{-4}$</td>
<td>$7 \times 10^{-4}$</td>
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</table>

* Approximate range of values taken from Table I.
† Approximate $K_i$ values calculated from the slopes of the Lineweaver-Burk plots presented in Figs. 13, 14, 16, and 17.
of magnitude as that of GDP instead of being 100 times greater as reported by Mazunder et al. (5). The higher apparent $K_m$ values reported by other workers might have been due to the facts that (a) the $K_m$ values for GDP and IDP were obtained by a coupled assay system in the presence of a product (CoA, 0.025 mg per 3 ml) and at a low concentration of succinyl-CoA (lower than the amount of CoA introduced into the system) (5), and (b) the $K_m$ values for CoA, ATP, and succinate for the spinach enzyme (4), and those for CoA, ITP, and succinate for the pig kidney cortex enzyme (5), were all determined by the hydroxamate method in which a high concentration of hydroxylamine was used. Kinetic data showing the highly inhibitory effect of hydroxylamine were presented in the Paper I of this series (8).

Studies of the inhibition of arsolenolysis by the normal reactants provide valuable insight into the nature of the binding sites on the enzyme. In the normal reaction, the apparent $K_m$ of succinate is of the same order of magnitude as that of phosphate, and these reactants appear to be mutually competitive at low concentrations but noncompetitive at high concentrations (Figs. 10, 11, and 12). In the arsolenolysis reaction, however, the inhibitory effect of succinate is remarkably less than that of phosphate, suggesting that arsenate shares a common binding site with phosphate, but not with succinate. This supports the postulate that succinate and phosphate, in the normal reaction, compete for a common intermediate (or a chemical bond), but not for a common binding site on the enzyme.

The fact that phosphate and GTP are strong competitors of arsenate in the arsolenolysis reaction suggests that arsenate, phosphate, and the terminal phosphate of GTP share a common binding site, thus excluding each other from binding to the enzyme. It is noteworthy that the inhibition constant of phosphate for the arsolenolysis reaction is remarkably lower than the apparent $K_m$ value of the same compound for the normal reaction, and that the inhibition constant of GTP is in the same order of magnitude as $K_m$ for the normal reaction. The inhibition constants of these compounds for arsolenolysis may be identical with the dissociation constants of the respective enzyme-substrate complexes for the normal reaction.

The initial velocity and product inhibition data presented in this paper are consistent with a random reaction sequence (presented schematically in Fig. 20) and with a rate equation derived for a similar reaction sequence (see "Appendix"). The rate equation is quadratic in terms of both succinate and phosphate concentrations, predicting that the plots of $1/v$ versus $1/[sucinate]$ and $1/v$ versus $1/[phosphate]$ are hyperbolas instead of straight lines. However, the possibility that this phenomenon (curved Lineweaver-Burk plots, Figs. 11 and 12) is due to the presence of two or more isoenzymes, to multivalency of the enzyme, or to other reasons cannot be ruled out at this time.

If the above reaction sequence is correct, and there is an obligatory chemical intermediate in which the bond energy of GTP or succinyl-CoA is preserved during the reaction catalyzed by the succinic thiokinase from pig heart, guanosine diphosphoryl-$S$-CoA (perhaps enzyme-bound) appears to be a more likely candidate than phosphoryl-$S$-CoA as proposed by Kaufman (4) for the spinach succinic thiokinase. A basis for this speculation is the presence, in the reaction sequence, of a complex which contains both GDP and CoA moieties, $E(GDP)(CoA)$ or $E(2TP)(CoA)(Suc)$ (or possibly $E(-P)(GDP)(CoA)(Suc)$). The reaction of this hypothetical intermediate with phosphate would lead to the formation of GTP and CoA; on the other hand, the reaction of this intermediate with succinate would lead to the formation of succinyl-CoA and GDP.

While the $K_m$ values of GDP and GTP for the normal reaction are of a similar order of magnitude, GDP is a much weaker inhibitor of arsolenolysis. This is consistent with the concept that arsenate and the terminal phosphate of GTP share a common binding site. The relatively weak but distinct competitive inhibition of arsolenolysis by GDP may be due to steric hindrance resulting from the larger molecular size of arsenate in comparison to phosphate. However, another possibility cannot be overlooked. If guanosine diphosphoryl-$S$-CoA (GDP-CoA) is an obligatory intermediate in the normal reaction carried out in the presence of GDP, arsenate and GDP would compete with each other for the thioester bond of succinyl-CoA; arsenate would form unstable arsenoyl-CoA, and GDP would make the intermediate, GDP-CoA. This would imply that, although arsenate and phosphate share a common binding site, they react with different intermediates, perhaps because of the larger size, the more metallic nature of arsenic than phosphate, or both. That arsenate and phosphate may react with different intermediates is supported further by the type of inhibition exhibited by arsenate when succinate is the variable substrate. If arsenate reacted with the same intermediate as phosphate, a noncompetitive rather than an uncompetitive inhibition should have been observed. The foregoing argument supports Kaufman's view of the mechanism of arsolenolysis (4).

Various isotope exchanges have been studied by previous workers with succinic thiokinase from various sources. Although certain of these data appear to disagree with the mechanism proposed above, a careful examination suggests that they may not necessarily be mutually exclusive.

For the phosphate-GTP exchange, the proposed mechanism predicts that the requirement for succinate should depend on the concentration of phosphate. At a low concentration of phosphate, the reaction pathway through $E.(GDP)(CoA)$ (Loop B in Fig. 20) would be predominant, and succinate would not be required for the exchange. However, as the concentration of phosphate increases, the pathway will shift to that through
the $E(GTP)(CoA)(Suc)$ complex, and succinate will be required for the exchange. This shift from one pathway to another seems to take place when the concentration of either succinate or phosphate is about 1 mM or greater, as suggested by the data presented in Figs. 11 and 12. Kaufman et al. reported that succinate was required for the phosphate-ATP exchange with the pig heart enzyme in the presence of 16.8 mM phosphate (14), and spinach enzyme in the presence of 5 mM phosphate (4). On the other hand, Mazumer et al. (5) reported a slow but unmistakable exchange of $^{32}P$ between phosphate and ITP in the absence of succinate with the enzyme from kidney cortex in the presence of 5 mM phosphate. However, they attributed this exchange to the presence of impurity in the enzyme preparation.

For succinic thiokinase from spinach, Kaufman (4) reported that the $^{32}P$ exchange between ADP and ATP required only Mg$^{++}$ and enzyme, and that the $^4C$ exchange between succinate and succinyl-CoA did not require the addition of ADP, but was stimulated by the addition of phosphate. In contrast to these results, the reaction sequence postulated in the present paper predicts that a complete system is required for the GDP-GTP exchange, and that Mg$^{++}$ and GDP are required for succinate-succinyl-CoA exchange. These apparent inconsistencies between the reported data and the predictions may be resolved if one accepts the hypothesis proposed by Kaufman and Alivisatos (15) that the spinach enzyme contains a bound prosthetic group, GDP (or IDP), and catalyzes two enzymic reactions, nucleoside diphosphate kinase and succinic thiokinase. It was also reported that the succinate-succinyl-CoA exchange was studied with the enzyme from pig heart, and the results were similar to those for the spinach enzyme (see Kaufman (4), footnote, p. 154). However, without a detailed description it is difficult to rule out the requirement of GDP on the basis of this experiment, because guanine nucleotides could have been introduced into the system from an impure preparation of succinyl-CoA (cf. Fig. 1 of the preceding paper (8)).

The present hypothesis also suggests that the phosphate group, not the phosphorh group, is cleaved away from GTP by breaking the P-O bond adjacent to the $\beta$ phosphorus atom, and that $^{18}O$ exchange will occur between succinate and GDP, but not between succinate and phosphate. According to Cohn (16, 17), the cleavage of the P-O bond of ATP at a similar position has not been found in any of the transphosphorylase reactions studied. An exchange of $^{18}O$ between succinate and phosphate has been reported with rat liver mitochondria (18) and with a succinic thiokinase preparation from E. coli (19). In a crude system such as the mitochondrial preparation, it is very possible that $^{18}O$ phosphate is incorporated into ATP and the isotope is transferred to GDP by a reaction such as GMP + ATP $\leftrightarrow$ GDP + ADP, then to succinate by the succinic thiokinase reaction, thus making possible the apparent $^{18}O$ exchange between orthophosphoric and succinate. Therefore, it will be necessary to repeat the exchange study with an enzyme preparation completely free of other nucleotides and phosphate-transferring enzymes.

It is recognized that an enzymic reaction mechanism may not be considered as established on the basis of kinetic evidence alone. Furthermore, in view of the difficulty in correlating some of the prior exchange data (especially the $^{18}O$ studies) with the kinetic evidence reported above, a final conclusion on the reaction mechanism of succinic thiokinase from pig heart must be postponed until more evidence becomes available. In any case, attempts must be made to find a compatible explanation for both the kinetic and isotope exchange data.

**SUMMARY**

1. Initial velocity and product inhibition studies have been performed with succinic thiokinase purified from pig heart. The results suggested a reaction sequence which may be described as follows: two substrates, guanosine diphosphate and succinic coenzyme A, bind with the enzyme in a random sequence; then phosphate, the third substrate, is added onto the enzyme either before or after the first product, succinate, leaves; finally, the remaining products, guanosine triphosphate and coenzyme A, are released from the enzyme, again in a random manner.

2. Approximate apparent $K_m$ values for succinyl-CoA (1 to $6 \times 10^{-2}$ M), CoA (5 to $20 \times 10^{-4}$ M), GTP (3 to $10 \times 10^{-4}$ M), 8-azaguanosine diphosphate (3 to $11 \times 10^{-6}$ M), 8-azaguanosine triphosphate (7 to $20 \times 10^{-4}$ M), succinate (4 to $8 \times 10^{-4}$ M), and phosphate (2 to $7 \times 10^{-4}$ M) were determined at various concentrations of the two other substrates, at an MgCl$_2$ concentration of 10 mM, and in the absence of known inhibitors, and were found to be considerably lower than those previously reported from other laboratories.

3. The ability of succinic thiokinase to catalyze the aroylanolysis of succinyl-CoA in the presence of Mg$^{++}$, and its inability to catalyze the aroylanolysis of GTP, are confirmed. The apparent $K_m$ value for aroylanolysis reaction (succinyl-CoA, 0.05 mM; MgCl$_2$, 10 mM; pH 7.4) is $1.0 \times 10^{-2}$ M. The apparent $K_m$ for succinyl-CoA (arase, 1 mM; MgCl$_2$, 10 mM; pH 7.4) is $1 \times 10^{-4}$ M.

4. GTP, phosphate, GDP, and succinate all compete with aroylanolysis. The $K_i$ values of phosphate ($3 \times 10^{-5}$ M) and GTP ($5 \times 10^{-6}$ M) are lower than the corresponding apparent $K_m$ values for the normal reaction. In contrast to this, the $K_i$ values of GDP ($4 \times 10^{-4}$ M) and succinate ($7 \times 10^{-2}$ M) are remarkably higher than the corresponding apparent $K_m$ values for the normal reaction. It is concluded that phosphate, aroylanol, and the terminal phosphate of GTP share on the enzyme a common binding site which is different from the binding site for succinate.

5. Guanosine diphosphoryl-S-CoA is proposed for consideration as the chemical intermediate (perhaps tightly bound to the enzyme) by which the bond energy of GTP or succinyl-CoA is preserved during the succinic thiokinase reaction.

**APPENDIX**

In a recent personal communication, Professor W. W. Cleland has pointed out that the data presented in this paper fit the mechanism shown in Scheme 1 and the corresponding rate equation.

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Ki,KaCPQR + K,K,KaBP + K,KABP + K,KABC

Ki,KaBCQ + K,K,ACR + K,K,AR + K,K,BPQ

Assumptions: k, > k, > k, > k,; k, > k,; k, > k,

Definitions of the kinetic constants:

Ki = k,k/k,k Ki = k,k/k,k Ki = k,k/k,k

K = k,k/k,k K = k,k/k,k K = k,k/k,k

Ki = k,k/k,k Ki = k,k/k,k Ki = k,k/k,k

Ki = k,k/k,k K = k,k/k,k K = k,k/k,k

Haldane and other relationships:


V, = k,k,E,E V, = k,k,E,E V, = k,k,E,E V, = k,k,E,E

where A = GDP, B = succinyl-CoA, C = phosphate, P = succinate, Q = GTP, and R = CoA.

The last four terms in the denominator of the rate equation (BCQ, ACR, BPQ, and APR) represent the formation of two "dead end" complexes, EAR and EBQ. It was assumed that the dissociation constant of Q from EBQ is the same as from EQ, and the dissociation constant of R from EAR is the same as from ER.

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