Succinate Thiokinase

VI. MULTIPLE INTERCONVERTIBLE FORMS OF THE ENZYME*

(Received for publication, April 7, 1972)

DAVID P. BACCANARI AND SUNGMAN CHA

From the Division of Biological and Medical Sciences, Brown University, Providence, Rhode Island 02912

SUMMARY

The techniques of isoelectric focusing and polyacrylamide gel electrophoresis have been used to demonstrate multiple forms of pig heart succinate thiokinase. There are at least five peaks of enzyme having isoelectric points of 6.4, 6.2, 6.0, 5.9, and 5.8; all are similar with respect to apparent molecular weight, heat stability, pH optimum, and reaction rates with substrate analogs. Also, two other forms with isoelectric points of 5.6 and 5.3 are seen under certain conditions.

Isotopic studies show that a major portion of the enzyme is phosphorylated in its native state, with the pI 6.2, 6.0, 5.9, and 5.8 peaks containing the same number of phosphates bound per unit of enzymic activity. The pI 6.4 form lacks exchangeable phosphate but can be converted to the pI 6.2, 6.0, and 5.9 forms by GTP. The pI 6.2 enzyme is converted to the 6.0 and 5.9 forms by the same treatment. When the pI 6.2 or 6.0 forms are incubated with coenzyme A, the pI 6.4 enzyme can be regenerated. Since the pI 6.4 enzyme contains neither exchangeable phosphate nor CoA, it has been called the free enzyme form. The free enzyme is unstable, losing up to 50% of its activity in 2 hours, but it may be protected by glycerol. The interconvertibility of the various enzymes is also seen when the pI 5.9 peak distributes to the other phosphorylated forms upon refocusing.

*This investigation was supported by Grant No. GB 30495 from the National Science Foundation and by the Life Insurance Medical Research Fund G-70-1. Taken in part from a dissertation submitted by Davide P. Baccanari to the Graduate School of Brown University in partial fulfillment of the requirements for the degree of Doctor of Philosophy. Preliminary reports (1, 2) were presented to the Federation Meetings in San Francisco, California, June 1971, and in Atlantic City, New Jersey, April 1972.
Enzyme Preparation—Succinate thiokinase was isolated from pig hearts by using the method of Cha (14, 15) except all buffers used in Steps 3 and 4 (dialysis of the ammonium sulfate precipitate and DEAE-cellulose column chromatography) contained 20% (v/v) glycerol. After purification, the enzyme was stored at 5° in 100 mM Tris-acetate buffer (pH 7.4) containing 20% glycerol. On occasion, the enzyme was concentrated by high pressure (50 p.s.i.) ultrafiltration using a membrane (Amicon PM-10) which retains proteins with a molecular weight greater than 10,000. Succinate thiokinase activity was measured at 30° by the spectrophotometric method previously described (17).

Isoelectric Focusing—Electrofocusing was carried out at 5° according to the method of Vesterburg and Svensson (18). The 20-ml cathode electrolyte, 250 mM NaOH in 60% sucrose, was routinely located at the bottom of the column. A 0 to 46% (w/v) sucrose density gradient, containing 1% ampholine carrier ampholyte of the desired pH range, was formed above the cathode and the 10-ml anode electrolyte, 360 mM sulfuric acid, completed the column. Enzyme was either included in the “light” solution before gradient formation, or a small volume was adjusted to the proper density and added directly to the column after three-fourths of the gradient was formed; identical results were obtained using either method. The total amount of salt present in the 110-ml volume was less than 0.5 mmoles and electrofocusing was usually completed in 40 hours, as determined by a constant power consumption of 0.3 to 0.5 watt at 600 volts. Electrofocusing for longer times did not improve the resolution of the enzyme peaks. Fractions containing 0.55 ml were collected at a flow rate of 1.1 ml per min and assayed for enzymic activity. Protein concentrations in peak tubes were estimated by measuring the absorbance at 280 nm and using the factor 0.9 absorbance unit per mg of protein. The pH of selected fractions was measured at room temperature in test tubes (10 X 75 mm). One determination generally took 10 to 15 min; these long time intervals were required for equilibrating the pH electrode with the viscous sample solution and often necessitated restandardization of the meter several times in the course of an experiment. This procedure made measuring the pH of these small volumes of viscous solutions the most difficult technical problem encountered.

Vertical Polyacrylamide Gel Electrophoresis—Analytical polyacrylamide gels (5%) were prepared and run in 75 mm Tris-acetate buffer, pH 8. Blank gels were first prerun at 300 volts for 5 hours, at 5°, with the buffer circulating between the anode and cathode compartments.

Stain for Succinate Thiokinase—An enzymic stain was developed to detect inorganic phosphate released in the succinate thiokinase reaction. After electrophoresis the gel was washed for 2 to 3 min in ice-cold buffer, then incubated at 37° for 1 hour in 50 ml of 60 mM Tris-succinate, pH 7.4, containing 10 mM MgCl2, 10 mM MnCl2, 0.4 mM GTP, and 0.4 mM CoA. Although at this concentration calcium inhibited the enzyme activity 70%, it was necessary for localizing the reaction product, inorganic phosphate. The gel was rinsed with distilled water for 1 min, then incubated at 37° for 30 min in 50 ml of a solution containing 80 mM maleic acid, 80 mM Tris base, 90 mM NaOH, and 3 mM lead nitrate. This process converted the calcium phosphate into lead phosphate. The slab was washed overnight in slowly running distilled water to remove traces of soluble lead, then immersed in 100 ml of 5% ammonium sulfide. The gels were washed in 5% acetic acid to clear the background, and succinate thiokinase appeared as brown lead sulfide bands. Bands were not seen when GTP was omitted or replaced by ATP or when Tris-succinate was replaced by Tris-acetate, indicating that the stain was specific for succinate thiokinase.

Molecular Weight Determinations by Sephadex Gel Filtration—A Sephadex G-100 column (2.5 X 50 cm) was prepared as described by Andrews (19). The calibration mixtures contained 1 mg each of RNase, chymotrypsinogen A, ovalbumin, creatine kinase, and aldolase in 1 ml of eluting buffers at various pH. Retention studies showed that two peaks of enzymic activity were observed on calcium phosphate gel-cellulose chromatography (14); one emerged with

![Fig. 1. Isoelectric focusing pattern of succinate thiokinase.](http://www.jbc.org/download/1.png)

**RESULTS**

Isoelectric Focusing—Electrofocusing partially purified succinate thiokinase (27 units per mg), in the pH range of 5 to 7, resulted in at least five peaks of enzymic activity (Fig. 1). Similar patterns were observed with many different preparations and with the enzyme isolated from a single heart. When highly purified enzyme (97 units per mg) was electrofocused, the specific activities of the pH 6.2, 6.0, and 5.9 peaks were 100, 109, and 104 units per mg, respectively. Although the pH of each peak varied up to ±0.05 pH unit between experiments, the enzymes will be called the pH 6.4, 6.2, 6.0, 5.9, and 5.8 forms. The pH 6.2 enzyme was the most abundant. Depending upon conditions, either the pH 6.4 or 6.0 form was seen in the second greatest quantity. The pH 5.9 and 5.8 enzymes were often present only in small, but detectable, amounts.

Purification of Succinate Thiokinase—It has been reported that two peaks of enzymic activity were observed on calcium phosphate gel-cellulose chromatography (14); one emerged with

![Downloaded from http://www.jbc.org](http://www.jbc.org/download/2.png)
the heme proteins and the other eluted at higher ionic strength. This phenomenon was examined further. Enzyme from both peaks was mixed and rechromatographed on a smaller column (2.5 x 20 cm), containing 15 g of cellulose and 400 mg of calcium phosphate equilibrated with 90 mM Tris-acetate buffer, pH 8. Only one symmetrical peak of activity emerged where the second enzyme peak from the large column was expected. Also, when each peak of the large column was electrofocused, both patterns were similar to that shown in Fig. 1. It appears, therefore, that the multiple enzyme peaks seen on calcium phosphate gel-cellulose chromatography were artifacts of the large preparatory column.

It has been reported that 25% of the enzyme activity of the pooled calcium phosphate gel-cellulose fractions was routinely lost upon ammonium sulfate precipitation and dialysis (14). If the dialysis buffer contained 20% (v/v) glycerol, this loss could be prevented (Table I). When the enzyme dialyzed against buffer containing 20% glycerol was electrofocused, the p1 6.4 peak appeared significantly larger than the one seen in Fig. 1, indicating this form was unstable without glycerol and probably denatured during purification. Two peaks of enzyme activity were also seen when a preparative was chromatographed on DEAE-cellulose (14). The first peak contained a small amount of enzyme, had a low specific activity, and was discarded in a routine purification. When a sample from this first peak was electrofocused (Fig. 2), only one major enzyme form with a p1 of 6.17 was observed. The same pattern was also seen when the enzyme was mixed with previously isolated p1 6.2 peak and electrofocused. These data illustrate that the enzyme form with the least net negative charge under the conditions of chromatography, pH 8, can be partially separated from the bulk of the enzyme. The p1 6.4 form was not seen when either of the enzymes eluting from the DEAE-cellulose column was electrofocused, because too little was present in this particular preparation.

Kinetic and Physical Parameters of Succinate Thiolkinase—The apparent \( K_m \) values for succinate, CoA, and GTP were measured to determine kinetic differences among the enzymes isolated by electrofocusing. Linear double reciprocal plots were observed with each variable substrate, and the kinetic parameters were calculated with a computer program developed by Cleland (20). The values for each form (Table II) were identical within experimental error and in agreement with those previously reported for the enzyme which was presumably a mixture of the various forms (21). However, the reciprocal plot with succinate as the variable substrate did not show a concave downward curvature as previously reported (21). A probable cause of this discrepancy will be discussed below.

The similarities in kinetic parameters among the electrofocused enzymes were further examined. When the reaction rates with substrate analogs were compared to the velocity of the standard
the addition of enzyme and the activity of each sample was expressed as percentage of the maximum value.

*Molecular Weight Determination*—Since there were similarities in physical and kinetic parameters, it seemed possible that the pattern seen in electrofocusing might have been caused by the dissociation or aggregation of enzyme subunits. If aggregation due to low ionic strength in the electrofocusing column was the cause of multiplicity, exposure to concentrated buffer would dissociate the protein and a heterogeneous pattern would be seen upon refocusing. However, the electrofocused pl 6.4, 6.2, and 6.0 enzymes were isolated, dialyzed against 100 mM Tris-acetate buffer, and still each showed, upon refocusing, only one major peak of activity with the original pH value. The apparent molecular weights of the electrofocused enzymes were determined on a Sephadex G-100 column at pH 8.0 and at the isoelectric point of each form. The results, listed in Table IV, show that each enzyme has a value of approximately 78,000; more importantly, there was no measurable activity in the fractions corresponding to one-fourth, one-half, two, three, or four times the molecular weight.

**Electrophoresis of Succinate Thiokinase**—Multiple forms of pig heart thiokinase have also been demonstrated by polyacrylamide gel electrophoresis. A heterogeneous sample was subjected to electrophoresis along with the isolated pl 6.4, 6.2, and 6.0 forms, then it was stained specifically for enzyme activity. As shown in Fig. 4, the native enzyme separated into two distinct bands; the one with the lowest mobility corresponded to an unresolved mixture of the pl 6.4 and 6.2 enzymes, and the other band had the same mobility as the pl 6.0 form. The figure also shows that the electrophoretic mobilities are consistent with the isoelectric points determined by electrofocusing.

**Identification of Phosphorylated Forms**—It has been previously reported that *E. coli* enzyme was isolated from the cell as a phosphorylated protein (22). Since up to 4 moles of phosphate can be bound per mole of pig heart enzyme (5), an obvious possibility was that the multiple peaks seen in electrofocusing were due to different numbers of phosphate covalently bound to the same enzyme molecule (i.e., 0, 1, 2, 3, and 4 moles of P_i per mole of enzyme). To test this possibility, the enzyme was incubated with ^32Pi, CoA, and MgCl_2. Under these conditions ^32Pi would exchange with any unlabeled phosphate already bound to the enzyme but would not generate new enzyme-phosphate bonds. The preparation was dialyzed thoroughly and electrofocused (Fig. 5). It can be seen that all peaks except the pl 6.4 enzyme were already phosphorylated in the native state. Since the specific activities of purified pl 6.2, 6.0, and 5.9 forms ranged from 100 to 109 units per mg, the previously determined value of 8.4 units of enzyme per mole (5) has been used to calculate the molar concentration of catalytically active enzyme. The merit of using this value is explained under "Discussion." Each of these forms contained up to 4 moles of exchangeable phosphate per 8.4 units of enzyme. The shoulder on the left side of the pl 6.2 peak was frequently observed and suggests a decreasing amount of phosphate bound per unit of active enzyme.

**TABLE IV**

*Estimation of molecular weights of electrofocused succinate thiokinase by Sephadex G-100 gel filtration*

The molecular weights of the various enzyme forms were determined at pH 8.0 using 100 mM Tris-acetate buffer containing 100 mM NaCl, and at a pH corresponding to the isoelectric point of each species using 100 mM potassium phosphate buffer containing 100 mM NaCl and 20% glycerol. The results at pH 8.0 were the average of two determinations. Duplicate values agree within ± 2000. The column was calibrated at both pH 8.0 and 6.0; essentially identical plots of elution volume versus log molecular weight were obtained.

![Graph](https://example.com/graph.png)

**TABLE III**

*Ratio of reaction rates between substrates and analogs*

The standard assay mixture contained 50 mM Tris-succinate (pH 7.4), 10 mM MgCl_2, 0.1 mM CoA, and 0.1 mM GTP. For the various experiments, 0.1 mM ITP or 0.1 mM dGTP was substituted for GTP or 1.25 mM itaconate (methylene succinic acid) was substituted for succinate acid.

<table>
<thead>
<tr>
<th>Enzyme species (pI)</th>
<th>Reaction rates</th>
<th>Ratio of reaction rates</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>dGTP:GTP</td>
<td>ITP:GTP</td>
</tr>
<tr>
<td>6.4</td>
<td>0.85</td>
<td>0.88</td>
</tr>
<tr>
<td>6.2</td>
<td>0.84</td>
<td>0.85</td>
</tr>
<tr>
<td>6.0</td>
<td>0.87</td>
<td>0.80</td>
</tr>
<tr>
<td>5.9</td>
<td>0.88</td>
<td>0.84</td>
</tr>
<tr>
<td>5.8</td>
<td>0.84</td>
<td>0.86</td>
</tr>
</tbody>
</table>

**Fig. 3.** Heat stabilities and pH optima of electrofocused succinate thiokinase. A, the pl 6.2 and 6.0 enzymes and the Peak 1 enzyme from the DEAE-cellulose column chromatography step were pooled separately and dialyzed against 100 mM Tris-acetate buffer (pH 7.4). Aliquots of each enzyme were placed in a covered test tube (10 X 75 mm), incubated at the indicated temperature for 5 min, then placed on ice. The samples were then assayed at 30° for enzyme activity and compared with preparations that had not been heat treated. B, the enzymes, isolated as above, were assayed spectrophotometrically with the reaction mixture maintained at various pH values. One milliliter of assay mixture contained in micromoles: sodium succinate, 50; GTP, 0.1; CoA, 0.1; MgCl_2, 10; and an appropriate amount of Tris base to give the desired pH. The reaction was started by the addition of enzyme and the activity of each sample was expressed as percentage of the maximum value.
Stability of pI 6.4 Enzyme—Early in this work the pI 6.4 enzyme was found to be unstable in Tris-acetate buffer, even at concentrations of 100 mM. Several “protecting” agents were examined for their stabilizing effect on the enzyme. Succinate, GDP, glycerol, dithiothreitol, and ampholine carrier ampholyte (pH 5 to 7) were tested, but only glycerol and ampholine protected to any significant degree. The pI 6.4 enzyme, stored in 100 mM Tris-acetate buffer (pH 7.4) containing 30% glycerol, retained 70% of its activity over a 2-month period. Buffer containing 20% glycerol adequately protected the enzyme for shorter periods of time and was routinely used in the DEAE column chromatography steps.

The pI 6.4 enzyme can also be protected through phosphorylation with GTP. In illustrating the instability of the pI 6.4 enzyme relative to the phosphorylated form, glycerol-protected pI 6.4 enzyme was divided into two aliquots; one was incubated with GTP for 10 min, then buffer was added to dilute each sample 50-fold so that the concentration of glycerol was no longer protective. Both samples were stored at 0°C and assayed periodically (Fig. 6). The phosphorylated enzyme was stable over the 4-hour duration of the experiment, whereas the nonphosphorylated form lost 50% of its activity in 140 min. The denatured enzyme could not be reactivated by GTP or dithiothreitol.

Formation of pI 6.4 Enzyme—Several investigators (6, 7) have
suggested that a high energy form of succinate thiokinase exists in which coenzyme A is covalently bound to the enzyme (E ~ CoA). Since the pI 6.4 form was generated when a partially purified preparation was incubated with CoA (e.g. Fig. 5), the possibility that this form of the enzyme might be E ~ CoA was examined. An enzyme preparation was incubated with [3H]CoA and MgCl₂ (conditions which promote the formation of the pI 6.4 enzyme) and electrofocused. The insignificant amount of H radioactivity associated with the newly formed pI 6.4 enzyme indicated that CoA was not bound. Therefore this peak has been tentatively identified as a “free” enzyme form, having neither exchangeable phosphate nor CoA.

It became of interest to determine the phosphorylated peak from which the pI 6.4 form was derived. The pH 6.2 enzyme was isolated, dialyzed against buffer, and a portion was refocused as a control (Fig. 7A). Another sample was incubated with CoA and MgCl₂, then electrofocused; it can be seen that the free enzyme was formed from the pH 6.2 peak by this treatment (Fig. 7B). When a similar experiment was carried out with isolated pI 6.0 enzyme (Fig. 8), an even larger percentage of the measured activity was found in the pI 6.4 peak. These data are consistent with previous findings showing that phosphate is released from phosphorylated succinate thiokinase during treatment with CoA (7).

Phosphorylation of pI 6.4 Enzyme—Since the pI 6.4 peak can be generated from both the pI 6.2 and 6.0 forms, the following experiment was performed to determine whether the same enzyme peaks are generated by phosphorylation of the free enzyme. The pI 6.4 enzyme was isolated, incubated with [3H]CoA, &GTP and MgCl₂, then refocused (Fig. 9). All of the enzyme was converted to the pI 6.4 peak by this treatment (Fig. 7A). Another aliquot was incubated with [8-¹⁴C, γ-³²P]-GTP and MgCl₂, then refocused (Fig. 9). Of the enzyme was added to the pH 6.0 enzyme before gradient formation and refocused for 45 hours with a final power consumption of 0.34 watt at 600 volts. B, the dialyzed pH 6.0 enzyme was concentrated by ultrafiltration before electrofocusing. The reaction mixture (20% glycerol) contained: CoA, 1 μmole; MgCl₂, 20 μmoles; and 17 units of enzyme in a final volume of 2.8 ml.

This experiment also indicates that the 32P radioactivity associated with the pI 6.2 and 6.0 peaks is not due to contaminating proteins, because all proteins with these isoelectric points and 0.9 nmole of guanine nucleotide per 8.4 units of enzyme. This experiment also indicates that the 32P radioactivity associated with the pI 6.2 and 6.0 peaks is not due to contaminating proteins, because all proteins with these isoelectric points...
were removed when the pI 6.4 peak was isolated. Upon incubating the pI 6.4 enzyme with labeled GTP and refocusing, it is highly unlikely that any protein other than succinate thio kinase could have changed isoelectric points to 6.2 or 6.0. A large amount of 14C radioactivity was associated with the pI 5.9 peak and represented up to 1.0 moles of guanine nucleotide bound per 8.4 units of active enzyme. This phenomenon was observed in several experiments, but the nature of the unusually high amount of ligand bound per unit of enzyme is not yet clear.

Interconversion of pI 6.2, 6.0, and 5.9 Enzymes—In addition to the interconversion of the various phosphorylated peaks through the free enzyme, other routes of interconversion might be possible. When the isolated pI 6.2 enzyme was incubated with [8-14C]GTP and MgCl₂, appreciable quantities of the pI 6.0 and 5.9 enzymes were observed (Fig. 10). No significant amount of radioactivity was localized with the pI 6.2 or 6.0 forms, but a large amount, corresponding to up to 7 moles of guanine nucleotide bound per 8.4 units of enzyme, appeared with the pI 5.9 peak.

A sample of pI 5.9 enzyme isolated in another experiment was concentrated by ultrafiltration (Diaflo, Amicon), incubated with MgCl₂, then refocused (Fig. 11). The pI 6.2, 6.0, and 5.8 enzymes were formed by this treatment, and a small peak of activity at pH 5.6 was also detected. This experiment was only performed once, because very small amounts of the pI 5.9 enzyme were obtained when the partially purified succinate thio kinase was electrophoresed. Until more of this form is accumulated it cannot be determined whether the observed transformations were due to magnesium, ultrafiltration, or a natural redistribution of enzyme forms. Nevertheless, the multiplicity of the pI 5.9 enzyme clearly demonstrates the interconvertibility of this form. The pI 5.0 form has been observed on several occasions, especially when large amounts of succinate thio kinase were electrophoresed, showing that it was a natural constituent of the heterogeneous enzyme.

In an attempt to generate other peaks from the pI 6.0 form, the isolated glycerol-protected enzyme was partially converted to the free form by CoA, dialyzed, then incubated with [8-14C]GTP. Upon electrophoresing, the pattern illustrated in Fig. 12 was observed. Little of the original pI 6.0 peak remained, and 14C-labeled guanine nucleotide was found to be associated with the pI 5.9 enzyme. In this particular experiment, a new major enzyme peak, which has never been seen before, emerged at pH 5.3. The nature of this enzyme is unknown and will be carefully studied in the future.

The experimental observations made in electrophoresing pig...
heart succinate thiokinase, including those already shown in Figs. 1 to 12, are summarized in Table V. This table illustrates protection of the unstable pH 6.4 enzyme by glycerol, the various conditions that promote peak interconversion, and the percentage of enzyme recovered after each experiment. In most cases 30 to 40% of the applied enzyme was lost in electrofocusing. Although the free enzyme form can be protected to some extent by ampholine carrier ampholytes, up to 60% of its activity was lost during electrofocusing. This accounts for the fact that the enzyme purified with glycerol and having a relatively large amount of free enzyme showed a low over-all recovery of 44% upon electrofocusing.

### Table V

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Treatment</th>
<th>Electrofocusing pattern</th>
<th>Total recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>Native, purified without glycerol</td>
<td>None (12)*</td>
<td>pH 6.4</td>
<td>pH 6.2</td>
</tr>
<tr>
<td>Native, purified with glycerol</td>
<td>None (8)</td>
<td>2</td>
<td>32</td>
</tr>
<tr>
<td>pH 6.4</td>
<td>None (1)</td>
<td>10</td>
<td>13</td>
</tr>
<tr>
<td>pH 6.2</td>
<td>GTP (3)</td>
<td>40</td>
<td>54</td>
</tr>
<tr>
<td>pH 6.0</td>
<td>None (5)</td>
<td>1</td>
<td>61</td>
</tr>
<tr>
<td>pH 5.9</td>
<td>GTP (2)</td>
<td>22</td>
<td>38</td>
</tr>
<tr>
<td></td>
<td>CoA (2)</td>
<td>10</td>
<td>25</td>
</tr>
<tr>
<td></td>
<td>None (3)</td>
<td>23</td>
<td>54</td>
</tr>
<tr>
<td></td>
<td>CoA (1)</td>
<td>33</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>CoA, GTP (2)*</td>
<td>3</td>
<td>23</td>
</tr>
<tr>
<td></td>
<td>MgCl₂, concentration (1)</td>
<td>61</td>
<td>66</td>
</tr>
</tbody>
</table>

* Numbers in parentheses represent the number of experiments performed.

† A band corresponding to the pH 5.3 enzyme was also seen upon electrophoresis.

**Discussion**

The technique of isoelectric focusing has been used to demonstrate that succinate thiokinase from pig heart can occur in at least seven different forms with isoelectric points of 6.4, 6.2, 6.0, 5.9, 5.8, 5.6, and 5.3. We have shown that the unstable pH 6.4 enzyme is devoid of both exchangeable phosphate and CoA, but it can be converted into the pH 6.2, 6.0, and 5.9 enzymes by phosphorylation with GTP. The pH 6.2, 6.0, 5.9, and 5.8 forms are not different in terms of bound phosphate per unit of enzyme activity, and all enzyme forms are interconvertible upon proper treatment.

In most of this study, partially purified preparations were used to minimize the selective loss of certain enzyme forms. For example, the pH 6.4 enzyme was found to be extremely unstable and little activity was seen upon electrofocusing unless the preparation was first protected by glycerol. This discovery not only made the present work more feasible, but also led to an improved purification procedure. The observed heterogeneity was not due to artifacts unique to the electrofocusing technique, as evidenced by the partial resolution of the enzyme by DEAE-column chromatography and the good correlation between the electrophoresis and electrofocusing patterns. Electrophoresis was not used extensively because of its poor resolution, the laborious staining technique, and the difficulty in preserving the enzymic bands.

Throughout this study, enzymic activity was used in calculating the number of moles of various ligands bound per mole of active enzyme. The molar concentration of succinate thiokinase was not estimated by protein weight because of the non-homogeneous nature of some preparations and the small amount of material used. The value 8.4 units per nmole was based upon past molecular weight estimations and numerous attempts to purify the enzyme to homogeneity; these preparations had specific activities of 90 to 110 units per mg when the protein concentration was estimated by absorbance at 280 nm. The highest specific activity ever observed was 120 units per mg, and the value 8.4 units per nmole was based upon this observation and a molecular weight of 72,000 (5). Therefore, there are some uncertainties associated with this value. For example, the extinction coefficient at 280 nm of purified succinate thiokinase may differ from that of the bovine serum albumin standard, and the specific activity of fully active homogeneous enzyme may be different from 120 units per mg. Also, catalytically inactive succinate thiokinase may still bind certain ligands, thus making the estimated number of ligands bound per mole of enzyme unrealistically high. Despite these uncertainties, or rather because of them, we are using the value 8.4 units of enzyme per nmole with the understanding that a minor correction may have to be made in the future when the protein chemistry (amino acid composition and subunit structure) of each enzyme form is studied.

Multiple forms of an enzyme may be caused by a variety of factors, as discussed in the recent IUPAC recommendation on nomenclature (23). In the course of this investigation these possibilities have been considered, and all but a few have been ruled out with varying degrees of confidence.

The interconvertibility of the various enzyme forms is a key factor in understanding succinate thiokinase polymorphism, in that it excludes those types of multiplicity that depend upon genetic variations and other irreversible processes. For example, the pH 6.4 enzyme appeared homogeneous when electrofocused in a narrow pH gradient (pH 5.7 to 6.7). Considering the wide pH differences separating the various phosphorylated forms, it is unlikely that the free enzyme represents a mixture of two or more proteins with different amino acid sequences that give rise to the pH 6.2, 6.0, and 5.9 peaks upon phosphorylation. In
addition both the pl 6.2 and 6.0 enzymes can be converted into other phosphorylated peaks. Therefore all of the forms are interconvertible, either directly or through the free enzyme, suggesting that the various forms of succinate thiokinase are not caused by differences in primary or quaternary structures as in many classical isozymes. Hydrolysis of the amide groups of asparagine and glutamine is known to cause polymorphism in other enzyme systems (24, 25). However, this process should be irreversible and is unlikely to be responsible for the polymorphism of succinate thiokinase.

Enzyme heterogeneity may also be due to different states of protein aggregation (26). This phenomenon does not appear to be a factor in succinate thiokinase polymorphism because the pl 6.4, 6.2, and 6.0 enzymes can all be isolated and recoupled as single peaks with molecular weights of approximately 78,000. Although some spontaneous transformations did appear (i.e. the pl 5.9 enzyme redistributed into several other forms upon refocusing), in most cases well defined conditions had to be met before such changes took place.

Another obvious possibility is that each enzyme form may represent a different enzyme-substrate compound. It is well established that the enzyme from various sources can be phosphorylated (7, 22), that the bound phosphate can exchange with free inorganic phosphate in the presence of CoA possibly through the formation of an ATP-CoA compound (5, 6, 27), and that the pig heart enzyme can bind up to 4 moles of phosphate per mole of the enzyme (5). Therefore, it was possible that the five most abundant forms seen in electrofocusing might represent the enzyme with 0, 1, 2, 3, and 4 moles of phosphate bound per mole of protein. The experiment shown in Fig. 5 demonstrated that the pl 6.2, 6.0, 5.9, and 5.8 forms were all phosphorylated, and the enzyme in each peak tube bound 4 moles of phosphate per 8.4 units of enzyme. The possibility that the nonphosphorylated pl 6.4 peak might be an E ~ CoA compound could be dismissed on the basis of the experiment with $^3$H-CoA, whereas experiments with $^8$Y2GTP indicated that differences in numbers of bound guanine nucleotide probably do not account for the enzyme heterogeneity, with the possible exception of the 5.9 form.

By examining the data as presented above, most of the common causes of enzyme multiplicity have been eliminated. It appears that the pl 6.4 peak arises from dephosphorylation of the phospho-enzymes, but the exact nature of the other forms is not known. An intriguing possibility is that the various phosphorylated enzymes represent conformational variations of a single protein, but larger quantities of each highly purified form must be obtained before this hypothesis can be tested. Also, since the pl 5.9 enzyme was converted into the other forms by incubation with MgCl2 and concentration by ultrafiltration, the possibility that binding of magnesium ion may be occurring, and thereby altering the isoelectric point of the enzyme, cannot be overlooked. Nevertheless, this report illustrates that not all multiple forms seen upon electrophoresis or electrofocusing are due to classical isozymes, and it also demonstrates a clear-cut separation of free and phospho-enzymes by their isoelectric points.

The occurrence of a major enzyme form with an isoelectric point of 5.3 is an interesting finding. This form was seen only after a phosphorylated enzyme was treated with coenzyme A then GTP (Fig. 12). Since the newly formed pl 5.3 enzyme did not have bound guanine nucleotides the possibility that this form may be either an E ~ CoA intermediate or a simple disulfide compound of the enzyme and CoA must be considered. In fact an extensive study of this form is presently being undertaken.

In the course of the present work, it became apparent that some of the old observations made in this laboratory must be reinterpreted. For example, it has been shown that the multiple peaks seen on calcium phosphate gel-cellulose chromatography (14) were artifacts, probably resulting from the low gel to cellulose ratio needed for rapidly processing 1.6 liters of crude extract. On the other hand, the peaks seen on DEAE-cellulose chromatography represented a partial separation of the pl 6.2 form from the bulk of the enzyme. Also, the free enzyme was found to be unstable and the purification procedure was modified to prevent its loss. The inclusion of glycerol in two steps of the purification resulted in doubling the enzyme yield while the specific activity was increased 3-fold over a comparable step in the method previously available (14).

In an earlier work (21), a discrepancy from Michaelis-Menten kinetics was reported for succinate. Namely, the reciprocal plot with succinate as the variable substrate was a concave downward curve. However in the present work, such a curvilinear plot has not been observed with either the crude enzyme or the isolated individual forms. A careful examination of the old records revealed a subtle difference in the experimental design. In the old experiments, the concentration of chloride ion as succinate was varied, whereas in the present experiments, the concentration of sodium ion changed with succinate. In each case the concentration of Tris was kept constant. When the standard assay mixture was prepared in increasing concentrations of NaCl or NH4Cl (10 to 100 mM), the enzyme reaction was progressively inhibited up to 35%. Sodium acetate or ammonium acetate (10 to 100 mM) did not inhibit the enzyme reaction, suggesting that chloride ion interferes with the reaction and is responsible for the curvilinear double reciprocal plots.

Acknowledgment—The authors wish to thank Mrs. Claire Bell for her fine assistance in the purification of the enzyme and in the molecular weight determinations.

REFERENCES
