Malate Thiokinase

EVIDENCE FOR A RANDOM SITE REACTION MECHANISM*

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Half-site reactivity in the malate thiokinase reaction was studied by measuring the reaction of enzyme-bound ligands in a series of single turnover experiments. A dimeric (αβ)2 enzyme form containing 13C succinyl-CoA on one αβ subunit pair and 3H]succinyl-CoA on the adjacent αβ subunit pair was prepared. Reaction of this enzyme species with ATP or inorganic phosphate resulted in the release of half of the bound succinyl-CoA. The succinyl-CoA released comprised a 50-50 mixture of [13C]- and [3H]succinyl-CoA. Likewise, enzyme containing 32P phosphate on one αβ subunit pair and nonradioactive phosphate on the adjacent αβ subunit pair reacted with ADP releasing half of the bound phosphate as a 50-50 mixture of radioactive and nonradioactive phosphate. These results serve to exclude an alternating site mechanism for the malate thiokinase reaction and support a random reaction of liganded subunits.

In addition, it has been shown that enzyme containing 1 phosphate/(αβ)2 dimer is inactive toward phosphate transfer. However, succinyl-CoA served to activate this enzyme species for phosphate transfer. These results can be explained in terms of subunit asymmetry. The simplest model is one in which subunit asymmetry is induced upon ligand binding.

Malate thiokinase (EC 6.3.1.9) catalyzes reversible ATP-dependent acyl-CoA formation with a number of dicarboxylic acids as substrates (1). The physiological substrate is L-malate (2, 3), although succinate can substitute equally well for malate in terms of both Vmax and Km (1). The enzyme as isolated exists as a tetramer of αβ dimers, (αβ)4; however, evidence has been presented that the catalytically active form of the enzyme is a dimer of dimers, (αβ)2 (4). The malate thiokinase reaction is similar to the succinate thiokinase reaction in that a phosphorylated enzyme (5-8) and succinyl phosphate have been implicated as reaction intermediates (8-13).

Most recently we have found that an acyl-CoA product of the reaction, succinyl-CoA, can form a tight noncovalent complex with the enzyme in which 1 succinyl-CoA is bound/αβ subunit (14). The enzyme form containing bound succinyl-CoA reacts with ATP resulting in the release of half of the bound succinyl-CoA with the concomitant phosphorylation of half of the available phosphorylation sites. This observation served as the basis for the suggestion that the malate thiokinase reaction exhibits half-of-the-sites reactivity when containing bound acyl-CoA (14).

Additional evidence for the existence of half-of-the-sites reactivity in the malate thiokinase reaction came from studies utilizing the active site-directed reagent methoxy carbonyl-CoA disulfide (15). This reagent reacts specifically with a thiol group at the acyl-CoA site of the enzyme. When all of the acyl-CoA sites are blocked the enzyme can still be phosphorylated by ATP and dephosphorylated by reaction with ADP. However, only half of the total phosphorylation sites are reacted (16).

In the present study a series of single turnover experiments have been conducted to further probe half-site-reactivity in the malate thiokinase reaction. The results of this study serve to exclude an alternating site reaction mechanism.

EXPERIMENTAL PROCEDURES

Malate thiokinase was purified from cells of Pseudomonas MA grown on methyamine as previously described (4). Cells were disrupted in a bead beater rather than in a French pressure cell. The purified enzyme, which exhibited a specific activity of 4.5-5.0 units/mg, was judged to be greater than 90% pure by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (17). The enzyme was routinely dialyzed against 20 mM Tes buffer, pH 7.4, prior to use.

Single turnover experiments were conducted by incubation of the appropriate enzyme form with radiolabeled substrate, followed by separation of the enzyme from free ligands by molecular sieving chromatography as previously described (14). In general, a 150-ml aliquot was chromatographed on a 1-ml column of Bio-Rad P-10 (100-200 mesh) according to the method of Penefsky (18). Controls were routinely run in which substrate alone was chromatographed under identical conditions. The separation of enzyme and free substrate was such that less than 0.1% of the free substrate co-eluted with the enzyme. The enzyme effluent from the P-10 column was analyzed for protein by the method of Bradford (19), and for enzyme-bound ligands by either determination of radioactive content by scintillation counting or of nonradioactive ligand by specific fluorometric assays. The specific activity of each of the enzyme forms isolated was found to be identical with that of the native enzyme.

Unless otherwise stated incubation of enzyme (generally 3-6 μM) and substrate was for 1 min at 4°C in 20 mM Tes buffer, pH 7.4. 13C]Succinyl-CoA was prepared as previously described (14), and purified by ion exchange chromatography. 2-Tritiated succinic acid was prepared from 2-tritiated α-ketoglutarate by the method of Zaman and Akhtar (20). The latter compound was prepared by the method of Rose (21).

14C]Succinic anhydride, 13C]Succinyl-CoA, T20, and 32P]ATP, and 32P]P, were purchased from New England Nuclear. Succinate thiokinase from Escherichia coli was a generous gift of Dr. J. Nishiuma, University of Texas Health Science Center at San Antonio.

Sucinyl-CoA was measured fluorometrically by coupling the succinate thiokinase reaction with the hexokinase and glucose-6-phosphate dehydrogenase reactions.
In order to confirm these experiments a second set of single turnover experiments was conducted in which the enzyme was initially phosphorylated by ATP yielding E-(P)2 (step 1, Table II). This enzyme form was reacted with [14C]succinate plus CoA producing enzyme in which both sites contained bound [14C]succinyl-CoA, E-(succinyl-CoA). The formation of E-(succinyl-CoA)2 presumably occurs via the intermediate formation of E-P-succinyl-CoA and yielded inorganic phosphate as a product identified by thin layer chromatography. E-(succinyl-CoA)2 was reacted with ATP to yield E-P-succinyl-CoA which was then reacted with tritiated succinate plus CoA. This reaction sequence yielded E-(succinyl-CoA)2 in which the newly formed succinyl-CoA contained tritium and the previously bound succinyl-CoA contained 3H. Reaction of this species with ATP resulted in a loss of 50% of the bound tritiated succinyl-CoA and 50% of the bound [14C]succinyl-CoA (step 5, Table II), thus confirming the results shown in Table 1.

In the experiments described in Tables I and II reaction of phosphoenzyme with labeled succinate plus CoA was assumed to yield enzyme-bound succinyl-CoA as judged by the incorporation of radioactivity into the enzyme. In order to confirm this E-P-succinyl-CoA was formed by reaction of E-(succinyl-CoA), with ATP, isolated, and incubated with tritiated succinate plus CoA. This should yield E-(succinyl-CoA)2 in which half of the bound succinyl-CoA is tritiated. After separation from free ligands, the enzyme was acidified to pH 2.0 by the addition of 10% trichloroacetic acid. Released succinyl-CoA was assayed fluorometrically, and correlated with the estimated enzyme-bound succinyl-CoA determined by the radioactive content of the enzyme. Results of this experiment showed that the enzyme contained 1 succinyl-CoA/α subunit as determined fluorometrically, and 0.5 succinyl-CoA/α subunit as determined by the radioactive content of the enzyme. This experiment demonstrates first that reaction of E-P-succinyl-CoA with succinate plus CoA leads to the formation of enzyme bound succinyl-CoA, and secondly that enzyme-bound succinyl-CoA does not exchange with free succinate in the presence of CoA.

It has been shown that fully phosphorylated enzyme (E-P3) reacts with succinate plus CoA to yield E-(succinyl-CoA)2, (14), presumably via the formation of E-P-succinyl phosphate.

### Table I

**Demonstration of the equivalence of reactivities of αβ subunit pairs containing bound succinyl-CoA—single turnover experiments initiated with E-(succinyl-CoA)2**

A series of single turnover experiments were conducted in which enzyme was incubated with substrate, separated from excess substrate and product by molecular sieve chromatography, and analyzed for bound ligands. The general procedures are given under "Experimental Procedures." Malate (20 mM αβ subunits) was initially incubated with 0.1 mM [14C]succinyl-CoA (specific activity 3 x 106 cpm/nmol) yielding E-(succinyl-CoA), as product (step 1). E-(succinyl-CoA), was reacted with 5.0 mM [γ-32P]ATP (specific activity 6.3 x 107 cpm/mmol) yielding E-P-(succinyl-CoA) (step 2), which in turn was reacted with 1.0 mM [3H]succinate (specific activity 9.0 x 106 cpm/nmol) plus 0.1 mM CoA (step 3). This regenerated E-(succinyl-CoA)2, in which prebound succinyl-CoA was labeled with 3H and newly formed succinyl-CoA was labeled with tritium. In the last turnover step 4), E-(succinyl-CoA)2 was again reacted with 5.0 mM ATP. This reaction sequence is depicted on the left side of the Table. In a separate experiment [γ-32P]ATP was used throughout to determine the extent of phosphorylation of the enzyme.

| Reaction step | Bound ligand | phosphate |
|---------------|--------------|-----------|
| 1. | [14C]succinyl-CoA | 0.98 | 0.98 |
| 2. | [14C]succinyl-CoA | 0.54 | 0.54 |
| 3. | [14C]succinyl-CoA | 0.45 | 0.48 |
| 4. | [14C]succinyl-CoA | 0.26 | 0.23 |
Malate Thiokinase: Random Site Reaction Mechanism

**TABLE II**

Demonstration of the equivalence of reactivities of αβ subunits containing bound succinyl-CoA—single turnover reactions initiated with E-(P)2

The same general experimental procedure described in the legend to Table I was employed. Malate thiokinase (30 µM in αβ subunits) was initially incubated with 1 mM ATP (step 1). A parallel experiment was conducted in which [γ-32P]ATP was used in order to assess the extent of phosphorylation of the enzyme. Isolated E-(P)2 was then reacted with 1 mM [14C]succinate (specific activity 3.15 × 10^6 cpm/nmol) and 0.1 mM CoA (step 2). The product E-(succinyl-CoA) was reacted with 5 mM ATP yielding E-P-succinyl-CoA (step 3), which after isolation was reacted with 10.0 µM [3H]succinate (specific activity 9.1 × 10^5 cpm/nmol) and 0.1 mM CoA (step 4). The enzyme product from step 4 contained 1 bound succinyl-CoA/αβ subunit of which the newly formed [14C]succinyl-CoA comprised half of the total succinyl-CoA while the previously bound [14C]succinyl-CoA comprised the other half. In the last step (step 5), the enzyme was again treated with 5 mM ATP, and after separation of the enzyme from free ligands, enzyme-bound succinyl-CoA was determined.

| Reaction step | [14C]succinyl-CoA | [3H]succinyl-CoA | Total succinyl-CoA | Phosphate
|---------------|-------------------|-----------------|-------------------|----------|
| 1. COO        |                   |                 |                   |<0.001 |
| 2. P-OCO-P    |                   |                 |                   |0.51     |
| 3. [14C]succinyl-CoA-OCO-OCO-P | 0.89 | 0.89 |                   |
| 4. [14C]succinyl-CoA-OCO-P | 0.51 | 0.51 |                   |
| 5. [14C]succinyl-CoA-OCO-P | 0.45 | 0.39 | 0.84 |
| P-OCO- succinyl-CoA | 0.22 | 0.24 | 0.56 |

**TABLE III**

Equivalent reactivities of αβ subunits containing bound succinyl-CoA reaction in the reverse direction

The same general protocol as described in the legend to Table I was utilized. Enzyme containing bound succinyl-CoA (E-(succinyl-CoA)) was prepared by incubation of enzyme (30 µM in αβ subunits) with 0.1 mM [14C]succinyl-CoA (specific activity 1.35 × 10^6 cpm/nmol) (step 1). After separation from free ligands, the enzyme was incubated for 1 min at 4°C with 5 µM inorganic phosphate (step 2). In a parallel experiment (32P), was utilized in this step and the resultant enzyme contained 0.48 nmol of bound phosphate/nmol of αβ subunit. The enzyme was next incubated with 1 mM [14C]succinate (specific activity 6.7 × 10^5 cpm/nmol) and 0.1 mM CoA (step 3), regenerating enzyme containing succinyl-CoA at both sites, [14C]succinyl-CoA as the newly formed succinyl-CoA and [14C]succinyl-CoA as previously bound succinyl-CoA. This enzyme form was again incubated with 5 mM inorganic phosphate and after separation from free ligands, the enzyme was assayed for bound succinyl-CoA.

| Reaction step | [14C]succinyl-CoA | [3H]succinyl-CoA | Total succinyl-CoA | Phosphate
|---------------|-------------------|-----------------|-------------------|----------|
| 1. COO        |                   |                 |                   |0.86     |
| 2. [14C]succinyl-CoA-OCO-P | 0.86 | 0.86 |                   |
| 3. P-OCO- [14C]succinyl-CoA-OCO-P | 0.51 | 0.51 |                   |
| 4. [14C]succinyl-CoA-OCO-P | 0.46 | 0.52 | 0.98 |
| P-OCO- succinyl-CoA | 0.26 | 0.26 | 0.52 |

and then E-P-succinyl-CoA as intermediates. The intermediate, E-P-succinyl-CoA, then reacts with succinate and CoA at the second site via similar intermediates. In order to test this hypothesis we made use of the known lability of succinyl phosphate. When E-P2 is reacted with succinate alone, and then the enzyme separated from free ligands, the resultant enzyme contains 0.5 phosphate/αβ subunit. The possibility that E-P rather than fully dephosphorylated enzyme was fortuitously isolated as a result of a slow reaction or a high Kₘ for succinate was ruled out since increasing either the reaction time from 1 to 10 min or the succinate concentration from 0.1 to 10.0 mM did not change the result. This observation demonstrates the stepwise reaction of each αβ subunit in the dimeric (αβ)₃ form of the enzyme. In this experiment reaction E-P2 with succinate yielded E-P, thus only one of the two phosphorylated sites reacted with succinate. The resultant enzyme form containing only one phosphate (E-P) appears inactive towards phosphate transfer.

Since the malate thiokinase reaction is reversible, it was also possible to conduct single turnover experiments in the reverse direction. E-(succinyl-CoA) was prepared from [3H] succinyl-CoA and then reacted with inorganic phosphate. This reaction resulted in the formation of E-P-succinyl-CoA not E-P2, showing that only half of the sites reacted with inorganic phosphate (Table III). Further treatment of E-P-[3H] succinyl-CoA with [14C]succinate plus CoA resulted in reaction at the phosphorylated site with the formation of E-(succinyl-CoA) labeled with 14C at one site and 3H at the other site. Reaction of this species with inorganic phosphate resulted in the release of half of the bound succinyl-CoA in which equal amounts of [3H]succinyl-CoA and [14C]succinyl-CoA were released.

The above experiments demonstrate randomness in the reaction at the succinyl-CoA sites. In order to investigate randomness in the reaction at the phosphate sites E-P2 was prepared by reaction of enzyme with [γ-32P]ATP. Incubation of E-P2 with either ADP or succinate resulted in reaction at only one of the two phosphorylation sites yielding E-P as a product (Table IV). Phosphorylation of the available site with ATP regenerated E-P2 with the newly phosphorylated site containing nonradioactive phosphate, and the previously phosphorylated site containing [32P]phosphate (a control experiment in which E-P was phosphorylated by [γ-32P]ATP confirmed phosphorylation of the enzyme). Reaction of this enzyme form with either succinate or ADP resulted in a loss of half of the bound phosphate. However, the isolated E-P form of the enzyme contained 50% of its original 32P content.
Thus, reaction of the fully phosphorylated enzyme is also random.

Reaction of E-P₂ with ADP results in the release of only one of the two bound phosphates. However, in the presence of added succinyl-CoA both bound phosphates are released. This observation could be interpreted to suggest that both sites of the dimeric enzyme must be occupied for reaction at the second phosphorylation site. To test this possibility, E-P₄-succinyl-CoA was isolated and then reacted with ADP. As shown in Table V, the presence of bound succinyl-CoA permits reaction at the second phosphorylation site. However, the release of phosphate from the enzyme is relatively slow (t₀.₅ ≈ 20 s at 4 °C). This slow phosphate transfer was not due to the release of enzyme-bound succinyl-CoA as confirmed in a separate experiment. However, reaction of ADP with E-P₄-succinyl-CoA was rapid when succinyl-CoA was included in the reaction mixture. These results suggest that succinyl-CoA may play a dual role in catalysis. Succinyl-CoA bound on an adjacent αβ subunit increases the rate of phosphate transfer while a second molecule of succinyl-CoA presumably binds to the phosphorylated subunit and provides an additional rate acceleration for the reaction.

**TABLE IV**
**Demonstration of equivalent reactivities of phosphorylated αβ subunits**

Phosphorylated malate thiokinase was prepared by incubation of enzyme with 0.5 mM [γ-³²P]ATP (specific activity 2.7 × 10⁶ cpm/nmol) (step 1). After isolation, the phosphorylated enzyme was incubated with 1 mM ADP to remove half of the bound phosphate (step 2). The enzyme was then rephosphorylated with 0.5 mM nonlabeled ATP (step 3), and after isolation reacted a second time with 1 mM ADP (step 4). The values in parentheses represent a parallel reaction in which [γ-³²P]ATP was used throughout the experiment.

| Reaction step | Radioactive phosphate nmol/nmol αβ subunit |
|---------------|-----------------------------------------|
| 1. 00         | 1.02                                    |
| 2. ³²P-00-00P  | 0.51                                    |
| 3. 00-³²P     | 0.49 (0.92)                             |
| 4. 00-³²P     | 0.24 (0.53)                             |

**TABLE V**
**Effect of bound and free succinyl-CoA on the reaction of E-P₄-succinyl-CoA with ADP**

Malate thiokinase containing bound phosphate and bound succinyl-CoA (E-P₄-succinyl-CoA) was prepared by reacting [γ-³²P]ATP with E-succinyl-CoA as described in the legend to Table I (steps 1 and 2). Reaction mixtures containing 4.6 μM enzyme (in terms of αβ subunits), 1 mM ADP, 2 mM MgCl₂, and 20 mM Tes buffer, pH 7.4, in a final volume of 0.1 ml were incubated at 4 °C for the times indicated. The reaction was terminated by the addition of 6 μl of 20% trichloroacetic acid and lyophilized to dryness. The lyophilized material was dissolved in 10 μl of water and chromatographed on polyethyleneimine cellulose using 0.5 M LiCl, 0.2 M HCOOH as the solvent system. The ATP and P₃ spots were identified by radioautography and cut out and counted. Recovery of radioactive phosphate was >90%. In one sample 0.1 mM succinyl-CoA was included in the reaction mixture.

| Reaction time s | Phosphate as E-P % |
|-----------------|-------------------|
| 0               | 100               |
| 20              | 43                |
| 30              | 47                |
| 40              | 39                |
| 10 (0.1 mM succinyl-CoA) | <10 |

**DISCUSSION**

The results obtained in this study confirm and extend our proposal that the malate thiokinase reaction exhibits half-of-the-sites reactivity. We have previously demonstrated that the dimeric form of the enzyme ((αβ)₂) containing bound succinyl-CoA at each site (E-(succinyl-CoA)₂) reacts with ATP at only one of the two potential phosphorylation sites. In the present study we have also shown that fully phosphorylated enzyme E-(P₂) reacts with ADP or with succinate at only one of the two potentially reactive sites.

In addition, the results of this study show that the dimeric enzyme containing bound ligands on each of the adjacent subunit pairs reacts in an interacting but random fashion. Thus, either αβ subunit pair has an equal probability for reaction, but once one subunit pair has reacted the adjacent subunit becomes unreactive. This finding serves to exclude an alternating site mechanism in which one αβ subunit pair reacts in the first turnover, while the adjacent αβ subunit pair reacts in the second turnover. An alternating site mechanism has been proposed for mitochondrial ATPase (22, 23) glycerolaldehyde-3-phosphate dehydrogenase (24), and malate dehydrogenase (25, 26). Cardon and Boyer (27) have recently pointed out the difficulties in establishing an alternating site mechanism in an enzyme reaction. However, in the present study the ability to isolate stable catalytic intermediates has permitted us to exclude an alternating site mechanism in favor of an interacting random site mechanism.

We have observed that fully phosphorylated enzyme reacts rapidly with ADP or succinate at one of the two available phosphorylated sites. The resultant enzyme which contains phosphate at one of the two available sites, although reactive in terms of repophosphorylation by ATP, is virtually inactive with respect to phosphate transfer to either succinate or ADP. The presence of succinyl-CoA bound to the enzyme greatly enhances the reactivity of the enzyme containing phosphate at one of the two available sites. Exogenously added succinyl-CoA increases the rate of reaction at the phosphorylation site even more. These data suggest that both αβ subunit pairs in the dimeric (αβ)₂ form of the enzyme must be occupied before phosphate can be transferred to an acceptor. Phosphate transfer of ADP is enhanced even further by the binding of succinyl-CoA to presumably the phosphorylated subunit. Thus, a putative intermediate in the phospho transfer reaction to ADP is succinyl-CoA-P-αβ-αβ-succinyl-CoA where the binding of succinyl-CoA to the phosphorylated αβ subunit pair is considerably weaker than the binding of succinyl-CoA to the nonphosphorylated αβ subunit pair.

The random reaction of the liganded αβ subunit pairs can be explained in terms of subunit asymmetry. Two models warrant considerations. The first model is one in which ligand binding induces asymmetry among subunit pairs, while the second model is one in which pre-existing asymmetry among αβ subunit pairs is inherent in the native enzyme. As illustrated in Fig. 1 both models can be used to explain the random reaction of αβ subunit pairs containing bound succinyl-CoA. In the pre-existing asymmetry model only one subunit conformation can be phosphorylated. In addition, in order for this model to accommodate the experimental data obtained in this study, there must be conformational equilibrium among the two asymmetrical subunits with an equilibrium constant of 1.0. In the induced asymmetry model the only assumption that need be invoked is that the conformation and hence the binding and reactivity of subunit pairs containing bound phosphate or tightly bound succinyl-CoA be different from nonliganded subunits.

A further restriction must be imposed in the pre-existing asymmetry model when trying to explain the random half-site
transfer of phosphate from fully phosphorylated enzyme to ADP. In order to account for transfer of only half of the enzyme-bound phosphate it must be postulated that either conformer equilibration does not occur with the half-phosphorylated enzyme, or that the half-phosphorylated enzyme is inactive toward phosphate transfer regardless of the subunit conformation. In the case of the pre-existing asymmetry model no additional assumptions need be invoked to explain the lack of reactivity of the half-phosphorylated enzyme. Since the phosphorylated subunit pair is adjacent to a nonliganded substrate pair, no induced asymmetry occurs to activate the enzyme-bound phosphate. It must be postulated that either transfer of phosphate from fully phosphorylated enzyme to ADP is ruled out, the experimental results obtained in this study can more simply be explained in terms of the induced asymmetry model.

Although the pre-existing asymmetry model cannot be ruled out, the experimental results obtained in this study can more simply be explained in terms of the induced asymmetry model.

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