Thiophosphorylation As a Probe for Subunit Interactions in *Escherichia coli* Succinyl Coenzyme A Synthetase

FURTHER EVIDENCE FOR CATALYTIC COOPERATIVITY AND SUBSTRATE SYNERGISM*

(Received for publication, April 14, 1983)

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Succinyl-CoA synthetase has an (αβ)2 subunit structure and shows half-of-the-sites reactivity with respect to the formation of the phosphohistidyl residues that acts as a catalytic intermediate. Adenosine 5′-O-(3-thio)triphosphate has been found to be a substrate, but the overall maximum velocity is 3 orders of magnitude lower than that seen with ATP. Moreover, steps of the reaction involving thiophosphoryl transfer are much slower than the corresponding phosphoryl transfers. These properties of adenosine 5′-O-(3-thio)triphosphate as a substrate have been exploited to test the concept of alternating sites catalytic cooperativity proposed earlier as a rationale for the subunit structure of succinyl-CoA synthetase. As predicted by this model for catalysis, the rate of discharge of thiophosphate from the enzyme in the presence of succinate and CoA is stimulated by ATP. Neither of two nonhydrolyzable analogs of ATP has an equivalent effect. The results indicate that the transfer of the thiophosphoryl group from the enzyme to succinate at one active site is not favored until the neighboring active site is phosphorylated by ATP, with accompanying reciprocal changes in the conformations of the two halves of the enzyme molecule.

One rationale for the prevalence of multisubunit structures in enzymes is embodied in the concept of catalytic cooperativity (1–3). According to this idea, catalysis at one active site may be promoted by conformational changes that result from the interaction of substrates with a neighboring active site. In its simplest terms, such a model could involve asymmetry in the configurations of otherwise identical subunits, with changes in structure of subunits that are transiently involved in different aspects of catalysis (e.g. covalent bond making and breaking, or attachment and release of products). Alternating sites catalytic cooperativity has been proposed to account for catalytic properties of such enzymes as the energy-transducing ATPases (1, 4, 5), glutamine synthetase (6, 7), glyceraldehyde 3-phosphate dehydrogenase (8), and the subject of this communication, succinyl-CoA synthetase (2, 3, 10, 11).

Succinyl-CoA synthetase from *Escherichia coli* catalyzes

\[
\begin{align*}
E + \text{ATP} &\Rightarrow E\cdot\text{PO}_4 + \text{ADP} \\
E \cdot \text{PO}_4 + \text{succinate} &\Rightarrow E \cdot \text{succinyl-PO}_4 \\
E \cdot \text{succinyl-PO}_4 + \text{CoA} &\Rightarrow E + \text{succinyl-CoA} + \text{P},
\end{align*}
\]

and the following reactions:

\[
\begin{align*}
E + \text{ATP} &\Rightarrow E\cdot\text{PO}_4 + \text{ADP} \\
E\cdot\text{PO}_4 + \text{succinate} &\Rightarrow E \cdot \text{succinyl-PO}_4 \\
E \cdot \text{succinyl-PO}_4 + \text{CoA} &\Rightarrow E + \text{succinyl-CoA} + \text{P}, \tag{3}
\end{align*}
\]

\[
\text{ATP} + \text{succinate} + \text{CoA} \Rightarrow \text{succinyl-CoA} + \text{ADP} + \text{P}, \tag{4}
\]

In reverse order, this constitutes the substrate-level phosphorylation step of the tricarboxylic acid cycle. The enzyme is a tetramer containing two subunit types and is thought to have an (αβ)2 dimer-of-dimers structure (2, 3, 12). It displays several unusual catalytic properties that must be reconciled with its subunit structure. These include "substrate synergism" (13), a term coined to describe the fact that the enzyme is fully active for catalyzing its partial reactions only when all substrate-binding sites are occupied. The active site is believed to bridge the α-β subunit contact (2, 3, 14, 15), and although one would expect to find two active sites per tetramer, succinyl-CoA synthetase shows half-of-the-sites phosphorylation by ATP: only one phosphoryl group is incorporated at any one time (2, 12, 16, 17).

The concept of alternating sites catalytic cooperativity has evolved for succinyl-CoA synthetase from work in this laboratory and elsewhere (3, 6, 11). According to this model for catalysis, the transfer of the phosphoryl group from *E*-PO4 to succinate and its subsequent displacement by CoA (Reactions 2 and 3 above) is promoted by the interaction of ATP with the other active site on the neighboring (αβ) half of the molecule. To test this prediction, measurements of the rates of single enzyme turnovers would be necessary, thus requiring the utilization of sophisticated rapid sampling techniques. An alternative to rapid kinetics is available if the reaction could be slowed sufficiently to permit sampling by conventional methods. To this end, we have found that ATP γS is a substrate for succinyl-CoA synthetase, and that the kinetic parameters of the ATP γS-based reaction are appropriate to test the model for catalytic cooperativity.

A previous report from this laboratory (3) described 31P-NMR experiments indicating that partial Reaction 2, the formation of enzyme-bound succinyl phosphate, is triggered at one of the two active sites by the phosphorylation by ATP of the neighboring site on the other half of the enzyme molecule. This proposal is strongly supported by the results presented herein, showing that the release of thiophosphate from the thiophosphorylated enzyme is facilitated by ATP. Thus, this study with independent approach and methodology

*The abbreviations used are: ATP γS, adenosine 5′-O-(3-thio)triphosphate; AMP-PCP, adenylyl-5′-yl (β,γ-methylene)diphosphate; AMP-PNP, adenylyl-5′-yl (β,γ-imido)diphosphate.

*Recipient of Medical Research Council Predoctoral Studentship and a research allowance from the Alberta Heritage Foundation for Medical Research.

† This work was supported by Grant MT-2805 from the Medical Research Council of Canada. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
provides confirmation of the operation of catalytic cooperativity among the αβ dimers of succinyl-CoA synthetase.

**EXPERIMENTAL PROCEDURES**

**Materials**—Succinyl-CoA synthetase was purified from *E. coli* (Crooks strain) grown on a phosphate-buffered, succinate-based medium essentially as described previously (17, 18). The concentration of the purified enzyme was determined from absorbance measurements at 250 nm (19). The activity of the enzyme was measured by the direct spectrophotometric method (20). The specific activity of the enzyme preparations used here was consistently near 45 units/mg. Coenzyme A (reference standard grade) and succinyl-CoA were obtained from P-L Biochemicals. ATP was purchased from Terochem Laboratories, Edmonton, Canada. ATPyS, AMP-PCP, and AMP-PNP were products of Boehringer Mannheim. (32)ATPyS was purchased from New England Nuclear Canada.

**Thiophosphorylation of Succinyl-CoA Synthetase**—The enzyme was thiophosphorylated at 21 °C in an incubation mixture consisting of 0.5 mM ATPyS (containing [32P]ATPyS of specific radioactivity of 600 Ci/mmol or greater), 10 mM MgCl₂, 60 mM Tris-HCl, pH 7.4, 2 mg/ml of succinyl-CoA synthetase, and where applicable 0.5 mM CoA (with 0.5 mM DTT) or 0.5 mM succinyl-CoA. Samples of 5 µl were removed at timed intervals and added to 20 µl of 0.2 M EDTA, pH 8.3, to stop the reaction. For the "zero time" sample, 5 µl was removed before the addition of the enzyme solution with subsequent correction for the difference in volume. Following the addition of 75 µl of 50 mM Tris-HCl to each sample to increase its volume, the protein was separated from free ligands and salts by the method of Penefsky (21) using 1-ml columns of Sephadex-G50 (fine) topped with a 1-mm thick layer of Dowex-CI (AG 1-X8, Bio-Rad), both equilibrated with 0.5 M Tris-HCl, pH 7.4. After centrifugation of the columns the entire eluant was taken for counting of radioactivity.

When the thiophosphorylation reaction had reached equilibrium (as evidenced by no further increase in the extent of enzyme thiophosphorylation), the remainder of the incubation mixture was desalted by the Penefsky method using a 5-ml column of Sephadex with a 1-cm layer of Dowex under the same conditions as described above. Removal of nucleotides from the thiophosphorylated enzyme was confirmed by measurement of the ratio of absorbance at 280 nm to that at 260 nm. Samples of thiophosphorylated enzyme were stored at 4 °C following radioactivity and enzyme activity measurements.

**Discharge of Thiophosphate**—32P-labeled thiophosphorylated succinyl-CoA synthetase was incubated at 21 °C in a 0.5-ml mixture consisting of 10 µl MgCl₂, 10 mM (Na)₅sucinate, 0.5 mM CoA, 0.5 mM DTT, 50 mM Tris-HCl, pH 7.3, and where applicable 1 mM ATP or 3.2 mM AMP-PCP or 2.4 mM AMP-PNP. The concentration of [32P]thiophosphorylated succinyl-CoA synthetase in the incubation mixture was approximately 0.4 mg/ml. At timed intervals, 80 µl samples were removed and the reaction therein was quenched with 20 µl of 0.2 M EDTA, pH 8.3. 32P-labeled thiophosphorylated succinyl-CoA synthetase was separated from [32P]thiophosphate by the Penefsky method (21) using 1-ml columns of Sephadex with a 1-mm thick layer of Dowex-Cl (AG 1-X8, Bio-Rad). Behaviour of the columns was determined by measurement of radioactivity in the lower layer of Dowex-Cl. The entire eluant was taken for counting of radioactivity. "Zero time" samples were obtained for counting of radioactivity. "Zero time" samples were obtained by taking an aliquot of the stock [32P]thiophosphorylated succinyl-CoA synthetase solution corresponding to the amount taken in the reaction mixture, and making up the volume to 80 µl where necessary before adding EDTA and carrying out the separation procedure. The reaction was started by the addition of [32P]thiophosphorylated succinyl-CoA synthetase.

The effectiveness of this methodology for estimating protein-bound thiophosphate was confirmed in several control experiments. Samples from the enzyme omitted control showed no counts above background, indicating that there was no spillover of labeled nucleotide into the protein fraction. This was confirmed in all experimental samples by measurement of the ratio of their absorbances at 280 and 260 nm. Furthermore, in the thiophosphate discharge experiments, controls showed that the labeled enzyme that was loaded onto the columns could be quantitatively recovered.

**Measurement of Radioactivity**—Radioactivity was measured by liquid scintillation counting using Aquasol (New England Nuclear Canada) with a Beckman LS-200 counter equipped with fixed-window isotope modules.

**RESULTS AND DISCUSSION**

**ATP₇S As a Substrate for Succinyl-CoA Synthetase**—A recent proposal for catalytic cooperativity between the subunits of succinyl-CoA synthetase (3) involves a model for catalysis in which the transfer of the phosphoryl group from the histidyl residue to succinate at one active site is stimulated by the phosphorylation of the active site located on the neighboring half of the molecule. The experiments described below are designed to test that prediction, but involve measurements of the rates of thiophosphoryl transfer since these are expected to be of much slower rate but otherwise equivalent mechanism to phosphoryl transfer reactions. Thus, the Michaelis constant for ATP₇S measured at saturating concentrations of succinate and CoA is 0.21 mM, only 1 order of magnitude higher than that measured for ATP (22). But the maximum velocity with ATP₇S (k₉₈ = 0.015 s⁻¹) instead of ATP (k₉₈ = 98 s⁻¹) is reduced some 6000-fold. These kinetic properties are appropriate to allow measurement of the kinetics of thiophosphoryl transfer to test the model for catalytic cooperativity.

**Thiophosphorylation of Succinyl-CoA Synthetase**—Fig. 1 shows the time course of thiophosphorylation of succinyl-CoA synthetase by ATP₇S. As was seen many times earlier for the phosphorylation reaction with ATP, thiophosphorylation also shows the half-of-the-sites behavior, reaching equilibrium when one of the two αβ halves of the molecule is thiophosphorylated. From the data of Fig. 1, one can calculate a first order rate constant for the thiophosphorylation reaction of 2.2·10⁻⁹ s⁻¹. At first glance this seems inconsistent with the participation of the thiophosphorylated enzyme as a catalytic intermediate in the ATP₇S-driven reaction, whose k₉₈ is 15·10⁻³ s⁻¹. It is clear, however, that this is another manifestation of substrate synergism: in earlier studies (13) it has been shown that the ATP:ADP exchange catalyzed by succinyl-CoA synthetase (representing Step 1 of the reaction) is slower than the net catalytic rate, but stimulated by the presence of all substrates at equilibrium or by succinyl-CoA alone (13). Similarly, a strong enhancement of the rate of thiophosphorylation occurs in the presence of succinyl-CoA (Fig. 2); the thiophosphorylation is complete within 30 s, indicating at least a 10-fold increase in rate. Significantly, the addition of CoA alone is without effect on the rate of thiophosphorylation.

Thus, as was proposed earlier on the basis of acceleration of exchange reactions (19) and partial reactions (3), the enzyme is most catalytically effective when all substrate binding sites are occupied, behavior characteristic of the property of substrate synergism.

In keeping with many previous demonstrations of half-of-the-sites phosphorylation of succinyl-CoA synthetase, the enzyme samples used in these experiments incorporated 1.01 ± 0.19 phosphoryl groups/αβ tetramer following incubation with excess ATP. In nine separate measurements on five different enzyme samples, the effectiveness of this methodology for estimating protein-bound thiophosphate was confirmed in several control experiments. Samples from the enzyme omitted control showed no counts above background, indicating that there was no spillover of labeled nucleotide into the protein fraction. This was confirmed in all experimental samples by measurement of the ratio of their absorbances at 280 and 260 nm. Furthermore, in the thiophosphate discharge experiments, controls showed that the labeled enzyme that was loaded onto the columns could be quantitatively recovered.

**Measurement of Radioactivity**—Radioactivity was measured by liquid scintillation counting using Aquasol (New England Nuclear Canada) with a Beckman LS-200 counter equipped with fixed-window isotope modules.

**FIG. 1. Thiophosphorylation of succinyl-CoA synthetase by ATP₇S.** For details see "Experimental Procedures."
different thiophosphorylation reactions, the number of thiophosphoryl groups incorporated per (αβ)₄ tetramer averaged 1.05 ± 0.08. Thus, except for rates, the phosphorylation and thiophosphorylation of succinyl-CoA synthetase appear to be equivalent processes. Both show half-of-the-sites behavior (only one of the two α subunits becomes labeled) and both are affected by substrate synergism. These observations lend credence to our strategy of using thiophosphoryl transfer as a probe for catalytic mechanism.

**Discharge of Thiophosphate**—According to the partial reactions outlined above (Equations 1–3), a single turnover reaction of E-PO₃ with succinate and CoA should result in the production of succinyl-CoA and the release of P, from the enzyme. However, the model for alternating sites catalytic cooperativity that has been proposed (3) predicts that the discharge of phosphate in the presence of succinate and CoA should be stimulated by the addition of ATP. This is because at least the step involving succinyl phosphate formation is believed to be stimulated by the phosphorylation of the active site on the adjacent half of the molecule. The kinetics of discharge of PSO₃⁻ from the thiophosphorylated enzyme is shown in Fig. 3. As predicted, the release of PSO₃⁻ in the presence of succinate and CoA is very slow; the single turnover reaction has a half-life of about 1 min, corresponding to a kᵢ of 1.07 x 10⁻² s⁻¹. ATP addition stimulates the rate of discharge of PSO₃⁻ from the thiophosphorylated enzyme, to the extent that the reaction is complete before the removal of the first sample at 10 s. In a separate control experiment (data not shown) the presence of ATP alone, without CoA and succinate, was shown to be insufficient for rapid thiophosphate discharge; only 12% of the PSO₃⁻ was released after 10 min. This result provides important confirmation for the contribution of catalytic cooperativity to the catalytic efficacy of succinyl-CoA synthetase. The concept has evolved in part from the observation that the production of succinyl phosphate from E-PO₃ and succinate can be detected by ³¹P-NMR only when ATP is present in the mixture (3). Significantly, this ability of ATP to promote Step 2 is not shared by the nonhydrolyzable βγ-methylene analog of ATP, AMP-PCP (3), strongly suggesting that it is phosphorylation of the adjacent site, rather than simple binding of ATP, that is responsible for the catalytic cooperative effect. The results presented in Fig. 4 support the same conclusion. This experiment shows that the addition of either AMP-PCP or AMP-PNP, both at saturating concentrations relative to their respective kᵢ values, provide little stimulation of the succinate/CoA-enhanced discharge of thiophosphate.

Communication between the two subunit types is clearly demonstrated by the synergistic effects that are transmitted from subsite to subsite within the overall active site that bridges the α-β contact. Occupancy of both the succinate and CoA subsites on the β subunit promotes the transfer of the thiophosphoryl group from ATPγS to the active site histidyl residue contained in the α subunit's contribution to the structure of the same active site (see Fig. 3). This is a clear manifestation of substrate synergism, complementing previous observations (3, 12, 13, 23). We also see strong reinforcement of the concept of alternating sites cooperativity as a rationale for the (αβ)₂ dimer-of-dimers structure. The ability of ATP, but not of its nonhydrolyzable analogs, to promote the dethiophosphorylation reactions (Steps 2 and 3) may be easily reconciled with a model in which phosphorylation of one active site provides a boost for catalysis of the rate-limiting step at the neighboring active site. Such a model is depicted in Fig. 5, the essence of which includes a phosphorylation-driven reciprocal change in the conformation of the two halves of the enzyme molecule. It is known that the...
phosphorylation of the \((\alpha\beta)_2\) structure is exergonic (16), but that the singly phosphorylated enzyme has a distinctly different conformation from that of the apoenzyme (17). The conformational change that is driven by monophosphorylation may produce a configuration at the neighboring site that is most effective for catalysis of the transfer of the phosphoryl group from the histidyl residue to succinate. The relationship of these events in time is not clear; for example, the phosphorylation could precede the structural rearrangement, giving a species of enzyme that is transiently bis-phosphorylated. Alternatively, the phosphorylation, rearrangement, and phosphoryl transfer at the neighboring site could occur in concerted fashion.

Malate thiokinase of Pseudomonas MA shares many features in common with succinyl-CoA synthetase, including an \((\alpha\beta)_2\) structure (24) and a phosphohistidyl-enzyme intermediate (25). This enzyme catalyzes the ATP-dependent formation of CoA thiosters of either malate or succinate. In the present context, it is of interest that malate thiokinase exhibits half-of-the-sites reactivity, but that the details of its behavior are different than that of succinyl-CoA synthetase. For example, the enzyme may be bis-phosphorylated by ATP, but only one of the two phosphoryl groups may be discharged by ADP or succinate (26). Binding of succinyl-CoA to the adjacent subunit appears to accelerate the reaction of E-PO\(_3\) with ADP, a result that is reminiscent of the catalytic cooperative behavior of succinyl-CoA synthetase described herein. Similarly, it has been shown that malate thiokinase can bind two molecules of succinyl-CoA very tightly, and that only one of these is released by the subsequent monophosphorylation of the enzyme (24). By a set of single turnover experiments, Hersh and Surendranathan (26) have shown that the ATP-driven release of succinyl-CoA occurs randomly rather than in an alternating fashion, but alternating sites cooperativity would not be expected for this situation in any event since both halves of the molecule bind succinyl-CoA simultaneously and tightly.

Proposals for alternating subunit action and catalytic cooperativity have been put forward for several other enzymes, and in some cases these ideas have been challenged (for review, see Ref. 9). As Cardon and Boyer have noted (9), catalytic cooperativity may be exploited in catalysis by many multisubunit enzymes, but the property may be generally refractory to experimental demonstration. The accumulated body of evidence is now particularly strong for its contribution to the efficacy of catalysis by succinyl-CoA synthetase.

Acknowledgments—We thank Dr. Louis B. Hersh for his assistance by sending us a copy of a manuscript (Ref. 26) prior to its publication.

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J. Biol. Chem. 1983, 258:14116-14119.

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