**Escherichia coli** Succinyl Coenzyme A Synthetase

**INHIBITION OF ATP-STIMULATED SUCCINATE ↔ SUCCINYL COENZYME A EXCHANGE AT LOW SUCCINYL COENZYME A CONCENTRATIONS BY AN ADP TRAP**

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The hypothesis that *Escherichia coli* succinyl-CoA synthetase functions by a cooperative alternating sites mechanism is based largely on the results of [$^{18}$O]phosphate exchange experiments (Bild, G. S., Janson, C. A., and Boyer, P. D. (1980) J. Biol. Chem. 255, 8109–8115). In those experiments, [$^{18}$O]P₁ ↔ succinate (predominantly) exchange appeared to proceed at greater rates (relative to the apparent amount of succinyl-CoA released from the enzyme) at low ATP in incubations containing ATP, CoA, succinate, [$^{18}$O]P₁, 0.48 mM hydroxyamine (as a succinyl-CoA trap), and a pyruvate kinase-lactate dehydrogenase ADP trap. The conclusion arrived at was that succinyl-CoA binding at one site was inversely related to ATP binding at the second site. Thus, the residence time of succinyl-CoA binding at a site would be longer at lower ATP concentrations.

Our experiments show that, under the incubation conditions described by Bild et al. (Bild, G. S., Janson, C. A., and Boyer, P. D. (1980) J. Biol. Chem. 255, 8109–8115), succinyl-CoA is not efficiently trapped. Thus, at ATP concentrations from 3.6 to 150 μM, concentrations of succinyl-CoA from 13 to 78 μM were observed. Succinate ↔ succinyl-CoA exchange reactions carried out in this range of ATP and subsaturating succinyl-CoA concentrations were found to be markedly inhibited by the addition of the ADP trap. This inhibition was more pronounced at higher ATP levels. At a saturating succinyl-CoA concentration (1.5 mM), addition of the ADP trap actually stimulated succinate ↔ succinyl-CoA exchange. Under these conditions, ATP ↔ P exchange was greatly depressed. These results are interpreted as follows. ADP is required for optimal binding of succinyl-CoA, but only when the latter is present at subsaturating concentrations; thus, the ADP trap inhibits the reaction. ATP exerts its stimulatory action on succinate ↔ succinyl-CoA exchange through a "other site" effect, i.e. in binding to the noncatalytic site of succinyl-CoA synthetase, it facilitates binding and release of succinyl-CoA at the catalytic site. ATP may also exert negative effects by inhibiting other site binding of ATP or by interfering with same site succinyl-CoA binding at subsaturating concentrations of the latter.

These data support the notion that a half-sites mechanism applies to succinyl-CoA synthetase, but suggest that the [$^{18}$O]P₁ ↔ succinate exchange data which have been instrumental in development of the cooperative alternating sites hypothesis should be re-evaluated.

Succinyl-CoA synthetase catalyzes the following reaction:

\[
\text{NTP + succinate + CoA} \xrightarrow{M^*} \text{NDP + P} + \text{succinyl-CoA} \quad (1)
\]

where \(M^*\) is a divalent metal ion and NTP and NDP are purine nucleoside tri- and diphosphates, respectively. The *Escherichia coli* enzyme utilizes the adenine, guanine, and hypoxanthine nucleotides, whereas the porcine heart enzyme uses only the guanine and hypoxanthine compounds (1). Another distinct difference between the two enzymes is that the bacterial succinyl-CoA synthetase is an \(\alpha\beta\) protein, consisting of two active sites, while porcine succinyl-CoA synthetase is an \(\alpha\beta\) protein containing one active site (2, 3).

The catalytic mechanism of succinyl-CoA synthetase is complex. However, the covalent intermediates in the reaction can be represented as shown in Equation 2, a–c (1).

\[
E + \text{NTP} \xrightarrow{a} E-P + \text{NDP} \quad (2a)
\]
\[
E-P + \text{succinate} \xrightarrow{b} E-\text{succinyl-P} \quad (2b)
\]
\[
E-\text{succinyl-P} + \text{CoA} \xrightarrow{c} E + \text{succinyl-CoA} + \text{P} \quad (2c)
\]

Recently, Bild et al. (4) proposed a cooperative alternate sites model for the \(\alpha\beta\) \(E. coli\) enzyme. Data consistent with the proposal have been reported by others (5, 6). In this model, it is hypothesized that product (succinyl-CoA) at one active site is released by the binding of substrate ATP at the other site. Succinyl-CoA synthesized at the second site is then released by binding of ATP to the first site. In this way, the two active sites are envisioned as generating product in an alternating fashion. The primary basis for this hypothesis is that oxygen exchange between \(^{18}\text{O}\) labeled inorganic phosphate and ATP and succinate appeared to proceed more rapidly at low concentrations of ATP. It was suggested that at low ATP concentration, release of bound succinyl-CoA is slower and therefore exchange of oxygen atoms of the latter and inorganic phosphate is more likely to occur. In these experiments, a pyruvate kinase system and 0.48 hydroxylamine were added to trap ADP and succinyl-CoA, respectively, as they were released from the enzyme. The assumption was made, therefore, that the levels of free succinyl-CoA and ADP in these experiments were negligible.

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We have observed that, at 25 °C, 0.48 M hydroxylamine is not an efficient trap of succinyl-CoA. It was therefore of interest to us to determine to what extent succinyl-CoA that had been produced in the forward reaction (see Equation 1), had been released from the enzyme, and had escaped reaction with hydroxylamine could contribute to partial reactions, such as succinate ↔ succinyl-CoA exchange, in analogy to oxygen exchange involving 18O-labeled inorganic phosphate.

EXPERIMENTAL PROCEDURES

Materials—Succinyl-CoA was purchased from P-L Biochemicals and CoA from Boehringer-Mannheim. ATP, NADH, phosphoenolpyruvate (trisodium salt), rabbit muscle pyruvate kinase, and bovine heart lactate dehydrogenase were obtained from Sigma. [1,4-14C]Succinate and [γ-32P]ATP were products of New England Nuclear. Polygram Cell 300 polyethyleneimine (0.1-mm thickness) sheets were purchased from Brinkmann Instruments. BioSolv aqueous solubilizer and 2.5-diphenyl oxazole were obtained from Beckman Instruments and Amersham/Searle, respectively.

Succinyl-CoA synthetase of E. coli was assayed and prepared, as described previously (7).

Radioactivity Assay—Aqueous samples were dissolved in 10% BioSolv (Beckman Instruments), 0.7% 2,5-diphenyl oxazole in toluene. Segments of thin layer chromatograms were counted in a toluene-based scintillation mixture. All counts were made in a Tracor Analytic Model 6892 scintillation counter.

Measurement of Succinyl Hydroxamate and 14C]Succinyl-CoA Remaining during Synthesis by Succinyl-CoA Synthetase in 0.48 M Neutral Hydroxylamine at Various ATP Concentrations—The incubation mixtures were made up with components added at essentially the same concentrations used by Bild et al. (4). The incubations were as follows: 60 mM Hepes-KOH (pH 7.2), 10 mM MgCl2, 20 mM dipotassium succinate, 0.19 mM CoA, 5 mM P~o, 1 mM phosphoenolpyruvate, 400 mM hydroxylamine hydrochloride (neutralized with KOH), 0.4 mM NADH, 3.6-150 µM ATP, 29 units/ml of pyruvate kinase, 63 units/ml of lactate dehydrogenase, and 0.4 µM succinyl-CoA synthetase. Incubation was performed at 25 °C.

For the determination of succinyl hydroxamate, the incubation mixture volume was 0.4 ml. Succinyl hydroxamate was quantitated after addition of an acid ferric chloride solution (See Ref. 7).

For the measurement of succinyl-CoA, the incubation mixture contained 2 µCi of 14C]succinate in a final volume of 0.1 ml. To stop the reaction, 50 µl of 0.1 M EDTA (pH 7.2) were added, followed by 1.9 ml of cold 0.003 N HCl. Four µl of 12.5 mM nonradioactive succinate, 10 mM MgCl2, 20 mM dipotassium succinate, 0.19 mM CoA, 5 mM P~o, 1 mM phosphoenolpyruvate, and 400 mM hydroxylamine hydrochloride (neutralized with KOH) were added to this mixture. Reaction mixtures were applied to thin layer chromatograms (see Ref. 7). Radioactivity was quantitated in both succinate (peak at fractions 7-19) and succinyl-CoA (peak at fractions 40-42) fractions, and the amount of succinyl-CoA was calculated on the basis of the percentage of radioactivity recovered in that fraction. The amount of nonradioactive succinyl-CoA was determined by subtracting the values obtained after addition of nonradioactive succinate from the values obtained after addition of radioactive succinate.

RESULTS

When succinyl-CoA was incubated with neutral 0.48 M hydroxylamine at 25 °C, the thioester bond, monitored at 235 nm, disappeared with a t1/2 of approximately 1 min (Fig. 1). In the first minute after initiation of the reaction, 19 nmol of succinyl-CoA was converted to succinyl hydroxamate. Thus, under these conditions, hydroxylamine cannot be considered an efficient trap of succinyl-CoA. In the experiments to be described, the levels of enzyme, ATP, succinyl-CoA, and CoA are such that the new succinyl-CoA is synthesized at a comparable rate.

It was therefore of interest to determine how much succinyl-CoA was present in the presence of hydroxylamine at the concentrations of ATP and incubation times employed by Bild et al. (4). The results of this study are shown in Table I. It is evident that, at the concentrations of enzyme and ATP used, significant quantities of succinyl-CoA were formed and the hydroxylamine trap simply could not keep pace with the synthesis of the thioester. The CoA concentration at the start of the incubation was 190 nmol/ml. Thus, at the higher concentrations of ATP, the concentrations of succinyl-CoA that could exist in the presence of hydroxylamine were significant. These data are plotted in Fig. 2 together with a time course of the formation of succinyl hydroxamate. It can be seen that, as ATP and phosphoenolpyruvate became exhausted, hydroxamate formation stopped abruptly and, since no new succinyl-CoA could be formed, its levels dropped precipitously, as expected. These results show that free succinyl-CoA could be a significant factor in the exchange of 18O and counted. The results were used to calculate the rate of exchange, according to the following relationship (8):

\[ \nu \text{(nmol exchanged/min)} = -\frac{XY}{X + Y} \ln(1 - F)/t \]

where X and Y are the concentrations of succinate and succinyl-CoA, respectively, F is the per cent exchange observed divided by the per cent exchange at isotopic equilibrium, and t is the time in minutes.

Measurement of ATP ↔ P, Exchange Reaction—The composition of the reaction mixtures was the same as that described for the succinate ↔ succinyl-CoA exchange assay (with ATP and succinyl-CoA concentrations varied), except that [32P]Pi (116,000 cmp) and nonradioactive succinate were added. Incubation was carried out with 0.1 µM succinyl-CoA synthetase for 5 min at 24 °C. The reaction was stopped and samples spotted on polyethyleneimine sheets, as described for the succinate ↔ succinyl-CoA exchange. Ascending chromatography was performed in 0.52 M potassium phosphate, pH 5.5. P, migrated into the upper half of the sheet, while ATP remained in the lower half. The respective areas were cut out and counted. Following determination of radioactivity incorporated into ATP, ATP ↔ P, exchange rates were calculated as described above for succinate ↔ succinyl-CoA exchange.

| ATP | Time (min) | Succinyl-NHOH | Succinyl-CoA |
|-----|------------|---------------|--------------|
| µM  |            | nmol/ml       | nmol/ml      |
| 3.6 | 15         | 175           | 15.9         |
| 30  | 358        | 175           | 14.6         |
| 60  | 675        | 175           | 0.7          |
| 90  | 750        | 175           | 1.5          |
| 128 | 750        | 175           | 24.9         |
| 150 | 6.2        | 500           | 58.0         |
| 150 | 2.4        | 500           | 73.8         |

TABLE I

Succinyl-CoA levels measured in the presence of hydroxylamine at different ATP concentrations

The incubation conditions and methods of analysis are described under "Experimental Procedures." The incubation times (128, 16.8, 6.2, and 2.4 min) were calculated from the data of Bild et al. (4).

1 J. S. Nishimura and T. Mitchell, unpublished results.

2 The abbreviation used is: Hepes, N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid.
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Fig. 1. The stability of succinyl-CoA in the presence of hydroxylamine. Succinyl-CoA was incubated in 0.48 M hydroxylamine hydrochloride that had been adjusted to pH 7.2 with NaOH. The reaction was monitored at 25 °C in a Cary Model 14 recording spectrophotometer set at 235 nm.

Fig. 2. Time course of succinyl hydroxamate formation and succinyl-CoA remaining at 3.6 µM ATP. Some of these data were taken from Table I.

between inorganic phosphate and other ligands, particularly succinate. This point can be visualized by reference to the scheme in Fig. 3. The scheme explains how one might envision $^{18}$O exchange between P$_i$ and succinate via enzyme-bound succinyl-CoA as proposed by Bild et al. (4). If, however, this enzyme-bound succinyl-CoA most significantly arises from rebinding of succinyl-CoA that was formed in the reaction (seen from left to right in the scheme) and survived the presence of hydroxylamine, an additional variable must be considered. It was of interest to know what effect the increased levels of free succinyl-CoA might have on $^{18}$O exchange. Since the methodology to perform $^{18}$O exchange experiments was not available to us, we used the succinate ↔ succinyl-CoA exchange reaction in its place. While succinate ↔ succinyl-CoA exchange may proceed at a different rate than $[^{18}$O]P ↔ succinate exchange. Since the methodology to perform $^{18}$O exchange experiments was not available to us, we used the succinate ↔ succinyl-CoA exchange reaction in place. The results shown in Fig. 4 confirm this expectation. The concentrations of succinyl-CoA used were in the range of those found in the experiments described in Table I and Fig. 2. Other conditions were different out of necessity, e.g. the enzyme concentration was reduced to facilitate accurate measurements of exchange rates, succinate concentration was reduced so that measurement of the exchange with $[^{14}$C]succinate might be feasible and hydroxylamine and the pyruvate kinase-lactate dehydrogenase ADP trap were omitted.

A surprising result was observed when the ADP trap was included. This is illustrated in Fig. 5. These results show that...
succinate ↔ succinyl-CoA exchange was actually inhibited as ATP concentration was increased. Inhibition was dependent on the presence of both phosphoenolpyruvate and pyruvate kinase. It was determined in another experiment that lactate dehydrogenase and NADH were not required for inhibition of the reaction. It has also been found by us (data not shown) that an ADP trap consisting of creatine phosphate and creatine kinase inhibited the ATP-stimulated reaction as effectively as the pyruvate kinase-lactate dehydrogenase system.

A possible explanation for this inhibition is that binding of ADP that would be present when the pyruvate kinase system are normally present in the added ATP, but are removed by the ADP trap. Some credence to this notion is afforded by the data shown in Fig. 6. In this figure, it can be seen that the inhibitory effects of the pyruvate kinase system were observed at relatively low succinyl-CoA concentrations. However, at a saturating level (1500 μM) of succinyl-CoA, the presence of the ADP trap actually resulted in the stimulation of succinate ↔ succinyl-CoA exchange. Trace amounts of ADP that would be present when the pyruvate kinase system is omitted might be expected to stimulate succinyl-CoA binding. This is consistent with the hypothesis of Moffet and Bridger (10), based on kinetic data, that ADP binding must occur before Pi, and succinyl-CoA can be bound to succinyl-CoA synthetase. At saturating levels of succinyl-CoA, ADP binding may not be critical in this respect.

Also illustrated in Fig. 6 is the great sensitivity of the ATP ↔ P exchange reaction at all three succinyl-CoA concentrations employed to the ADP trap at 1500 and 150 μM ATP. Since succinate ↔ succinyl-CoA exchange was actually stimulated by the ADP trap at the highest succinyl-CoA concentration, it would appear that the participation of ATP in stimulating the succinate ↔ succinyl-CoA exchange reaction might involve an other site, rather than a same site, effect.

**DISCUSSION**

The data described in this paper point to the strong possibility that the results described by Bild et al. (4) are explainable in great measure by the fact that succinyl-CoA is not instantaneously consumed by 0.48 m hydroxylamine at 25 °C. Thus, a significant part of [3H]P, ↔ succinate exchange that occurs in the presence of hydroxylamine may be ascribed to rebinding of surviving low levels of succinyl-CoA to the enzyme. As shown in Table I and Fig. 2, these levels are not trivial. In fact, at 3.6 μM ATP, we have seen that a steady state level of about 13 μM succinyl-CoA was maintained over a period of 60 min. Since succinyl-CoA has a greater apparent affinity for the enzyme than ATP (1), it is not difficult to conceive that, under these conditions, a significant part of enzyme-bound succinyl-CoA was probably derived from the rebinding of succinyl-CoA that survived in the presence of hydroxylamine. At 20 and 50 μM ATP, at least equilibrium concentrations of succinyl-CoA were found and, even at 150 μM ATP, the amount of succinyl-CoA detected was again striking. *A priori*, these data would not seem to qualitatively affect the results reported by Bild et al. (4). However, the marked inhibition of ATP-stimulated succinate ↔ succinyl-CoA exchange by an ADP trap (Fig. 5) strongly indicates that binding of succinyl-CoA at low concentration to succinyl-CoA synthetase is significantly dependent upon the presence of ADP. This ADP, which is an unavoidable contaminant of ATP, would be expected under these conditions to be quantitatively reconverted to ATP. It was not surprising, therefore, that at higher ATP concentrations, greater inhibition was observed since, with ADP removed, the higher levels of ATP could compete more effectively with succinyl-CoA for same site binding. It is possible that in the absence of an ADP trap, a delicate balance exists between succinyl-CoA binding and reactivity and the ATP/ADP ratio. This may have physiological significance, particularly in view of the observation made by Murakami et al. (11) that succinyl-CoA stimulates an apparent purine nucleoside diphosphate kinase activity of succinyl-CoA synthetase.

The observation that the ADP trap does not inhibit succinate ↔ succinyl-CoA exchange at saturating succinyl-CoA, but actually stimulates it, may have served to pinpoint the mechanism by which ATP acts as a positive effector of this reaction. At saturating succinyl-CoA, it would not be unreasonable to predict that succinate ↔ succinyl-CoA (refer to Equation 2, b and c) would proceed in the absence of ADP. What then is the role of ATP in stimulating this exchange reaction? It is unlikely that ATP and succinyl-CoA would be bound simultaneously at the same active site. Thus, it would seem that other site binding of ATP in stimulating the suc-
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cinate $\leftrightarrow$ succinyl-CoA exchange reaction of succinyl-CoA synthetase would best explain the data. The inhibition of ATP $\leftrightarrow$ P$_i$ exchange at higher ATP (Fig. 6) is consistent with a negative interaction between sites, i.e. binding of ATP at one site inhibits binding at the other site. It would seem puzzling that ATP $\leftrightarrow$ P$_i$ exchange, which should be representative of the overall reaction (Equation 2, a-c), should proceed more rapidly than succinate $\leftrightarrow$ succinyl-CoA exchange (see rates at 150 $\mu$M ATP, Fig. 6). This is not difficult to rationalize, since ATP release and binding from the enzyme could occur at faster rates than those of succinyl-CoA.

In conclusion, the data reported in this paper support the notion that site-site interaction occurs in E. coli succinyl-CoA synthetase. Thus, ATP binding at one site affects the rate at which chemical events and (or) binding events occur at the other site. However, the data also cast some doubt that the conclusions arrived at by Bild et al. (4) regarding an alternating sites mechanism for succinyl-CoA synthetase were based on valid data. The cogent experiments of Surendranathan and Hersh (12) with malate thiokinase, an enzyme that is analogous in mechanism to succinyl-CoA synthetase, have provided evidence for a random site mechanism in that case.

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