Dephosphorylation of Succinyl Coenzyme A Synthetase as Related to Enzyme Specificity and Catalytic Intermediates*

J. L. Robinson, R. W. Benson, and P. D. Boyer†

ABSTRACT: With succinyl coenzyme A synthetase from *Escherichia coli*, additions of coenzyme A did not result in dephosphorylation of ^{32}P-phosphoenzyme or the formation of enzyme-bound coenzyme A in the presence of ^{32}P-labeled adenosine triphosphate. This gives further evidence that an enzyme-bound coenzyme A form is not a catalytic intermediate. The succinate-promoted dephosphorylation of ^{32}P-phosphoenzyme requires succinate concentrations considerably above the apparent K_m for succinate or the concentration of succinate required for maximal decrease of the K_m for coenzyme A. The inability of succinate to dephosphorylate completely ^{32}P-phosphoenzyme and the very slow initial rate of dephosphorylation are not consistent with the formation of an enzyme bound succinyl phosphate as an independent step in the catalysis.

A concerted mechanism is suggested for further consideration. Specificity studies using the enzyme dephosphorylation assay show a catalytic activity of 4-phosphopantetheine but none for the coenzyme A analog with O replacing S. Of 11 organic acids tested, only succinate, malate, and to a much lesser extent fumarate and α-ketoglutarate showed activity as measured by dephosphorylation of ^{32}P-phosphoenzyme. In the net catalysis, malate at high concentration was about 3% as effective as succinate.

Succinyl-CoA synthetase in presence of its substrates is phosphorylated on the N-3 position of a histidine residue (Hultquist *et al.*, 1966) and the phosphoenzyme, E-P, meets kinetic criteria for an intermediate in the catalysis (Bridger *et al.*, 1968). The phosphoenzyme can arise directly from phosphorylation by ATP, or by an unknown mechanism from P_i in the presence of succinyl-CoA. The preceding paper (Benson *et al.*, 1969) presents quantitative ^{18}O evidence that an enzyme-CoA form, such as participates in a CoA transferase reaction (Hersh and Jencks, 1967; Benson and Boyer, 1969), does not participate in the principal catalytic pathway of the synthetase reaction. Two other prominent possibilities are the participation of an enzyme-bound succinyl phosphate, as suggested by Nishimura and Meister (1965) and Nishimura (1967), or a concerted formation of the phosphohistidine, analogous to the suggestions of Buchanan (1959) and of Buchanan and Hartman (1959) for the over-all reaction of some ATP-linked syntheses.

The present paper reports examination of the effects of CoA, succinate, and other compounds on the dephosphorylation of E-P. Such studies give a sensitive measure of enzyme specificity and a valuable assessment of possible catalytic intermediates. The sensitivity of the approach derives from the fact that it measures only one turnover of the catalytic intermediate, E-P. The results obtained are inconsistent with the participation of an enzyme-CoA form as an intermediate, and are difficult to reconcile with but do not eliminate participation of an enzyme-bound succinyl phosphate as an intermediate. In addition to the assessments of possible catalytic intermediates, results are presented using the E-P dephosphorylation assay on specificity of the enzyme for CoA-related substances and for various organic acids.

Materials and Methods

Enzyme Preparation and Assays. Succinyl-CoA synthetase was purified from succinate-grown Crookes strain *Escherichia coli* (Miles Laboratories) and assayed spectrophotometrically as described by Ramaley *et al.* (1967). E-P was formed by incubation of enzyme with ATP or with succinyl-CoA plus P_i, followed by Sephadex isolation (Ramaley *et al.*, 1967). E-^{32}P was measured as phenol-extracted counts after stopping the reaction by addition of liquefied phenol or shaking with phenol-saturated water (Ramaley *et al.*, 1967). To remove any CoA derivatives with the enzyme, a solution of enzyme was passed through a column of anion-exchange resin AG-1 (Cl^-), (Bridger *et al.*, 1968).

Substrates and Assays. CoA and ATP were purchased from P-L Biochemicals. $^{32}P_i$, ATP-γ-^{32}P, and succinyl-^{14}C-CoA were prepared, purified, and determined as described by Ramaley *et al.* (1967). CoA was measured by an adaptation of the fluorometric assay of Garland *et al.* (1965) as described elsewhere (Benson *et al.*, 1969). 4-Phosphopantetheine and oxy-CoA (Miller *et al.*, 1966) were the kind gift of Dr. Charles J. Stewart of the San Diego State College. The malate used was recrystallized from diethyl ether.

Results

Substrate Specificity for CoA. If the requirement of CoA for dephosphorylation of E-P in presence of succinate or for the slow E-P $\rightleftarrows$ P_i exchange in absence of succinate reflected only binding of the CoA and not a participation or an influence of the SH group in the catalysis, oxy-CoA (the CoA analog

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TABLE I: The Ability of 4-Phosphopantetheine and of Oxy-CoA to Substitute for CoA.[a]

Additions	E-^{32}P Formed (mole/mole of enzyme)	Succinyl Ester Formed (μmoles/min mg of protein)
None	0.04	0
1 mM oxy-CoA	0.02	$<3 \times 10^{-5}$[b]
10 mM 4-phosphopantetheine	0.65	0.02
0.1 mM CoA	0.83	17

[a] For measurement of exchange of E-P with $^{32}P_i$, 19 μM E-P was incubated with 36 μM $^{32}P_i$, 0.1 M Tris-Cl (pH 7.4), 0.1 M KCl, and 0.01 M $MgCl_2$ for 60 min at 25°. Net succinylation of CoA and of phosphopantetheine was measured spectrophotometrically as described by Ramaley *et al.* (1967). Measurement of possible net formation of succinyl oxy-CoA was made under the same conditions, but with use of succinate-^{14}C, and with separation of succinate and possible succinyl-oxy-CoA on a DEAE-cellulose column as described by Moyer *et al.* (1967). [b] Estimate of sensitivity limit; none detected in 3 hr.

TABLE II: The Ability of Various Organic Acids to Promote Dephosphorylation of E-P in Presence of CoA.[a]

Organic Acid Added	% of E-^{32}P Remaining
None control minus CoA	100
None	81
Succinate	11
Malate	15
α-Ketoglutarate	37
Fumarate	39
Maleate	81
Citrate	73
Acetate	76
Malonate	75
Glutamate	78
Aspartate	79
Acetoacetate	81

[a] For each sample, E-^{32}P (5.4 μM, specific activity 8) in 0.1 M Tris-Cl (pH 7.2), 0.1 M KCl, 10 mM $MgCl_2$, 10 μM CoA (except as indicated), and 1 mM of the organic acid (total volume 0.11 ml) was incubated for 25 min at 25°. The reactions were stopped by the addition of 5 ml of water-saturated phenol, and E-^{32}P was extracted into phenol (Ramaley *et al.*, 1967).

with an O replacing the S) might be expected to substitute for CoA. Essentiality of the SH group could be reflected by activity of portions of the CoA molecule, such as 4-phosphopantetheine. These substances were tested both by the ability to promote the E-P $\rightleftarrows$ P_i exchange and by the possible net formation of their succinyl derivatives in presence of ATP and Mg^{2+} as shown in Table I. The ability to stimulate the E-P $\rightleftarrows$ P_i exchange is the more sensitive measure, and the catalytic capability of 4-phosphopantetheine is readily revealed by this assay. In the net synthesis assay, the activity of 4-phosphopantetheine was only roughly $^1/_{1000}$ that of CoA. No ability of oxy-CoA to replace CoA could be detected by either assay.

Substrate Specificity for Succinate. The capacity of different organic acids to promote enzyme dephosphorylation was of interest both as related to the specificity of the catalytic site and for assessment of the possible role of succinyl phosphate as a catalytic intermediate. Results of experiments with different organic acids are given in Table II. Besides succinate, only malate, α-ketoglutarate, and fumarate promoted dephosphorylation of E-P in presence of added CoA. The other carboxylic acids tested showed no dephosphorylation above that observed with CoA alone. The small release with CoA alone likely reflects exchange of E-^{32}P with the trace contamination of P_i.

A slow dephosphorylation occurred in presence of succinate and malate even in the absence of CoA. The time course for dephosphorylation by malate, fumarate, or malonate was measured, as shown in Figure 1. Only malate gave an appreciable dephosphorylation rate. The observed maximum turnover rate, about 0.17/min, is approximately one-fifth that given by succinate under comparable conditions. This is a greater relative activity than observed when malate was tested as a substrate for net thiol ester formation in the usual enzyme assay. As tested at a relatively high concentration of 91 mM malate, the rate of thiol ester formation observed was 3% of that with succinate.

The dephosphorylation of E-^{32}P by carboxylic acids appeared to depend upon protein structure. As shown in Table III, in the presence of 6 M urea, succinate, malate, or malonate did not promote enzyme dephosphorylation. Also shown in Table III is the inhibition by EDTA of the dephosphorylation of E-^{32}P by succinate. This likely reflects a requirement of Mg^{2+} for the reaction.

The Apparent Michaelis Constants for Succinate and CoA. The concentration dependence of the net catalytic reaction upon succinate is relevant to the tests on the ability of succinate to dephosphorylate E-P. A dependency of the apparent K_m for succinate upon the CoA concentration was noted, and thus limited measurements were made on the effects of both CoA and succinate on the net reaction velocity. The results given in Table IV show a marked dependency of the K_m values for succinate or for CoA upon the concentration of the other substrate. The apparent K_m values for succinate are two to three orders of magnitude higher than those for CoA. Increase in the succinate concentration from 10 to 100 mM did not, within experimental error, further decrease the K_m for CoA. The concentration of succinate required for half-maximal decrease of the K_m for CoA is thus less than 10 mM. The K_m for succinate at the lowest CoA concentration used (10 μM) was 4.3 mM. Experimental difficulties obviated measurement of the K_m for succinate at CoA concentrations lower than 10 μM.

Lack of CoA-Stimulated Dephosphorylation of E-P. If CoA reacted with E-P to give an enzyme–CoA intermediate as de-

TABLE III: The Ability of Urea and EDTA to Inhibit the Dephosphorylation of E-P by Organic Acids.[a]

Addition	% of E-^{32}P Remaining
None	100
Urea	103
Urea plus malate	99
Urea plus succinate	99
Urea plus malonate	95
Succinate	28
EDTA plus succinate	90

[a] For each sample, E-^{32}P (2.1 μM, specific activity 23) in 0.1 M Tris-Cl (pH 7.3), 0.1 M KCl, 10 mM $MgCl_2$, and 6 M urea as noted was incubated 5 min at 37° to allow temperature equilibration. Then malate, succinate, malonate, or EDTA was added as indicated to give concentrations of 91 mM (final volume 0.11 ml) and the samples were incubated for 10 min. The reactions were stopped by adding 5 ml of water-saturated phenol, the E-^{32}P was extracted into phenol.

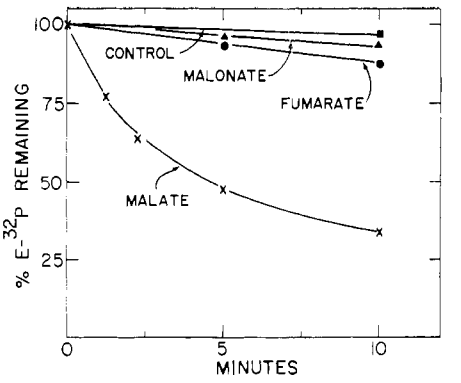


FIGURE 1: The ability of malate, fumarate, and malonate to dephosphorylate E-P in absence of CoA. For each sample, E-^{32}P (2.1 μM, specific activity 23) in 0.1 M Tris-Cl (pH 7.3), 0.1 M KCl, and 10 mM $MgCl_2$ was incubated for 5 min at 37° to allow temperature equilibration. The enzyme used had previously been passed through ion exchange resin to remove traces of CoA (see Methods). Then malate (x), malonate (▲), or fumarate (●) was added to 91 mM (final volume 0.11 ml). No additions were made to one sample (■). At the times indicated, the reactions were stopped by adding 5 ml of water-saturated phenol and E-^{32}P was extracted into phenol.

picted by eq 1, a release of $^{32}P_i$ and formation of a bound CoA

$$AT^{32}P + E \underset{ADP}{\rightleftarrows} E\cdot{}^{32}P \underset{CoA}{\rightleftarrows} E\ CoA + {}^{32}P_i \quad (1)$$

should occur under appropriate conditions. The equilibrium of enzyme with ATP favors E-P formation (Ramaley *et al.*, 1967), and thus addition of ATP-^{32}P converts a high portion of the enzyme into the E-^{32}P form. Figure 2 shows the effects of addition of CoA to enzyme that had been phosphorylated with ATP-^{32}P. Neither a definitive increase in $^{32}P_i$ nor a marked decrease in E-^{32}P was observed after CoA addition. The slow increase in $^{32}P_i$ over a 50-min period probably reflects a very slow exchange between E-^{32}P and the small amount of P_i (about 0.02 mM) present as a contaminant in CoA; the E-^{32}P level is likely maintained by exchange with the ATP-^{32}P present at about ten times the contaminating P_i level.

To further assess if the very slow $^{32}P_i$ release might reflect formation of an enzyme-bound CoA, direct measurement was made of CoA bound to the protein and released by appropriate incubation with excess P_i. The enzyme protein in the reaction mixture as used for the experiment reported in Figure 2 was separated on a Sephadex column and measurements of E-^{32}P and CoA were made as outlined in Table V. The results show that the ^{32}P with the enzyme was readily released by incubation with CoA, succinate, and Mg^{2+}, or by exchange with excess P_i in presence of added CoA. The ^{32}P-enzyme thus remained catalytically active, and any bound CoA would be released upon incubation with excess P_i and Mg^{2+}. A low amount of free CoA was present with the enzyme as measured

TABLE IV: Apparent Michaelis Constants for Succinate and Coenzyme A.[a]

Varied Substrate	Fixed Substrate Concn	App K_m
Succinate	10 μM CoA	4.3 ± 0.7 mM
Succinate	100 μM CoA	1.5 ± 0.1 mM
CoA	1 mM succinate	29.0 ± 2.0 μM
CoA	10 mM succinate	8.7 ± 1.1 μM
CoA	100 mM succinate	7.6 ± 0.7 μM

[a] The K_m values were calculated from appropriate initial velocity measurements using a computer program based on the statistical method of Wilkinson (1961). Each K_m was calculated from ten or more individual assays of net catalytic activity at 28° in 0.1 M Tris-Cl (pH 7.3), 0.1 M KCl, 10 mM $MgCl_2$, 0.40 mM ATP, and various concentrations of succinate and CoA (see Robinson, 1968).

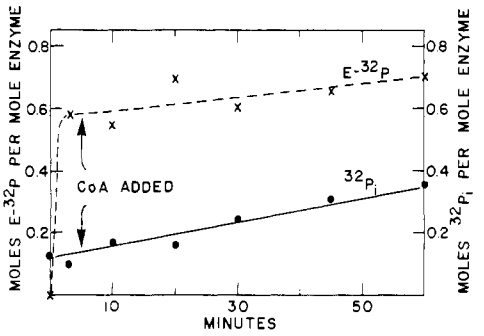


FIGURE 2: Lack of CoA-stimulated dephosphorylation of E-P. Enzyme (12 μM, specific activity 14) was incubated in 0.1 M Tris-Cl (pH 7.3), 0.1 M KCl, 10 mM $MgCl_2$, and 0.19 mM ATP-γ-^{32}P (final volume 5.0 ml) for 5 min near 0°. Then CoA was added to a final concentration of 10 mM (P_i contamination 0.02 mM) and the mixture incubated for 55 more min at 25°. At the times indicated, aliquots were removed for $^{32}P_i$ (●) and for E-^{32}P (×) determinations as described in Methods.

TABLE V: Measurement of ^{32}P and CoA Present with E^{32}P Treated with CoA.[a]

Preparation	Moles/Mole of Enzyme-^{32}P	Moles/Mole of Enzyme-CoA
As isolated	0.70	0.07
After reaction with CoA (1 mM), succinate (10 mM) and $MgCl_2$ (10 mM), 15 min at 25°	0.02	
After reaction with CoA (1 mM), P_i (1 mM), and $MgCl_2$ (10 mM), 15 min at 25°	0.05	
After reaction with P_i (0.25 mM) and $MgCl_2$ (10 mM), 15 min at 25°		0.07

[a] Enzyme catalysis in the incubation mixture described with Figure 2 was stopped after 60 min by cooling to 0° and adding EDTA to 0.1 M. The reaction mixture was placed on a G-50 Sephadex column (1 × 90 cm) previously washed with 0.1 Tris-Cl (pH 7.3), 0.1 M KCl, 10^{-4} M EDTA, and the enzyme was eluted with the same buffer at 5°. The eluent was collected with an automatic fraction collector and the enzyme-containing fractions were identified by A_{280}. Aliquots of these fractions containing 1.8 mμmoles of enzyme were removed for the characterization of phosphoryl (E-^{32}P) and free CoA as described in Methods. Each value represents the average of duplicate analyses.

by the sensitive fluorometric assay. However, the amount of detectable CoA was not increased upon addition with excess P_i and Mg^{2+}. The results thus give no evidence for formation of an enzyme-CoA form with properties as expected for a catalytic intermediate.

The Succinate-Dependent Dephosphorylation of E-^{32}P. As noted by Nishimura (1967) with the *E. coli* enzyme and by Butler (1964) and Cha *et al.* (1967) with the beef heart enzyme, succinate will cause a dephosphorylation of E-P. If such dephosphorylation reflects the formation of an enzyme-bound succinyl phosphate as an independent catalytic step, a concentration dependence, rate, specificity, and reversibility consistent with the properties of the net over-all catalytic reaction might be observed. The assay used for measurement of dephosphorylation of E-^{32}P was by measurement of ^{32}P extracted with the enzyme protein into phenol. This assay measures total conversion of E-^{32}P into $^{32}P_2$ and to succinyl-^{32}P (whether enzyme-bound or in solution) at the time of phenol addition. All ^{32}P found in the phenol extract has properties of phosphohistidine. The E-^{32}P used in these experiments was purified by passage through an anion-exchange resin (AG 1-Cl). Such treatment removes CoA or other substances, and makes the E-P → P_2 exchange completely dependent upon CoA (Ramaley *et al.*, 1967). Presence of CoA would complicate the experiments on dephosphorylation by succinate.

Figure 3 shows the rate and extent of dephosphorylation o E-^{32}P in presence of increasing concentrations of succinate up to 91 mM. The dephosphorylation pattern is obviously complex, particularly at the highest succinate concentration where a biphasic response is clearly evident. That the enzyme retained the capacity for complete dephosphorylation is demonstrated by the disappearance of E-^{32}P upon addition of CoA to samples containing succinate.

The dephosphorylation of E-P, as noted in Figure 3, requires a relatively high succinate concentration. Similar high succinate concentrations were used by Nishimura (1967). The initial rate of dephosphorylation in our experiments, even at the highest succinate concentration tested (91 mM), corresponds to a turnover only of about 0.8/min, and this rate soon decreases sharply to about 0.02/min. If the biphasicity of the dephosphorylation noted in Figure 3 results from the formation of an E-succinyl-P, which slowly dissociates or hydrolyzes, then E-P and succinate must be in equilibrium with E-succinyl-P. By dilution of the succinate, E-P should be regenerated as the concentration of the free succinate is drastically decreased. Figure 4 shows, however, that upon a nearly 100-fold dilution followed by 5-min additional incubation, the levels of E-P before and after dilution are the same within experimental error. No evidence for reversibility of the dephosphory'ation of E-P by succinate was obtained. This result shows that any succinyl-P that might be present in the reaction mixture has apparently dissociated from the enzyme.

Discussion

The inability of additions of CoA to cause dephosphorylation of E-^{32}P or to form protein-bound CoA in presence of ATP-^{32}P (Figure 2 and Table V) is not consistent with the formation of an E-CoA derivative. The findings are in harmony with the report by Cha *et al.* (1967) with the heart enzyme showing that binding of CoA was much less than discharge of bound phosphate. These results thus support the conclusion from quantitative ^{18}O-balance studies (Benson *et al.*, 1969) that an enzyme-bound CoA form does not participate in succinyl-CoA synthetase catalysis. In previous experiments from this laboratory (Moyer *et al.*, 1967), an ability of CoA addition to dephosphorylate E-P was noted. Their conditions were such, however, that trace contaminants of P_i or ATP in the CoA preparations at a concentration about equimolar with the enzyme could cause loss of ^{32}P from the E-^{32}P and thus apparent dephosphorylation of the enzyme. P_i and ATP or ATP-like impurities that are difficult to remove are present in commercial CoA preparations (Robinson, 1968). In the experiment as reported in Figure 2 this difficulty is obviated by the continued presence of a ATP-^{32}P to maintain an E-P in the ^{32}P form. In addition, the analyses for possible protein-bound CoA (Table V) rule out the possibility that both a ^{32}P and a CoA were bound to the same enzyme.

The pronounced specificity of dephosphorylation of E-^{32}P for succinate (Table II and Figure 1) and the apparent dependence on protein structure (Table III) support the view that the dephosphorylation reflects a specific binding of succinate at the catalytic site. Such specificity is consistent with the suggestion (Nishimura, 1967; Nishimura and Meister, 1965) that an enzyme-bound succinyl phosphate is an intermediate in the catalysis. That a protein-bound succinate at a saturable site is responsible for the dephosphorylation is also indicated by the decreasing dependence of the rate and extent of dephosphorylation upon succinate as the concentration is increased (Figure 3). The concentration of succinate required for half-maximal rate of initial dephosphorylation (obviously

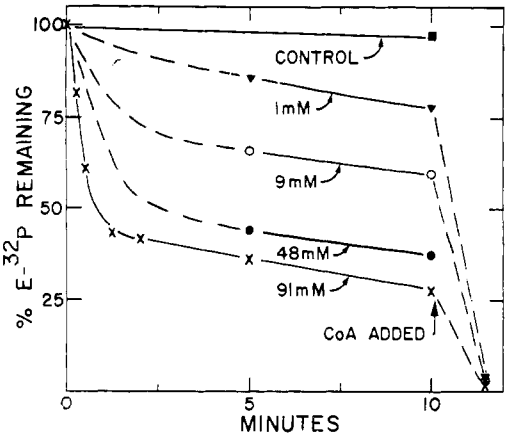


FIGURE 3: Dephosphorylation of E-P by increasing concentrations of succinate. For each sample, E-^{32}P (1.9 mM, specific activity 24) in 0.1 M Tris-Cl (pH 7.3), 0.1 M KCl, and 10 mM $MgCl_2$ was incubated for 5 min at 37° to allow temperature equilibration. Then succinate was added at the following concentrations: 0 (■), 1 (▲), 9 (○), 48 (●), and 91 mM (×) (total volume 0.11 ml). CoA (1 mM) was added to samples after 10 min. At the times indicated, the reactions were stopped by adding 5 ml of phenol-saturated water and E-^{32}P was extracted into phenol.

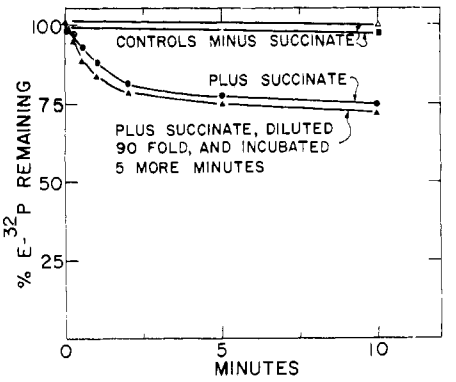


FIGURE 4: Lack of reversibility by dilution of the succinate-dependent dephosphorylation of E-P. For each sample, E-^{32}P (2.4 μM, specific activity 23) in 0.1 M Tris-Cl (pH 7.3), 0.1 M KCl, and 10 mM $MgCl_2$ was incubated for 5 min at 37° to allow temperature equilibration. Then succinate was added to 9.1 mM (total volume 0.11 ml). Some reactions (●) were stopped at the indicated times by adding 5 ml of phenol-saturated water. Other samples (▲) were diluted at the indicated times by adding 10 ml of a solution containing 0.1 M Tris-Cl M Tris-Cl (pH 7.3), 0.1 M KCl, and 10 mM $MgCl_2$, incubated 5 more min, and stopped by adding 2 ml of liquified phenol. To other samples, no succinate was added; one such sample was incubated without dilution (■) and another was incubated following dilution (△); these were stopped with 5 ml of water-saturated phenol or 2 ml of liquified phenol, respectively. E-^{32}P was determined as described in Methods.

>10 mM from results given in Figure 3) is considerably above the K_m for succinate (4.3 mM) at the lowest concentration of CoA tested. The unusually high concentration of succinate required for the dephosphorylation does not have adequate explanation. The concentration dependency for dephosphorylation is not readily comparable with the K_m for succinate or the succinate concentration required for maximal influence on the K_m of CoA because the kinetics are obviously complex and inadequately explored interrelations among substrates exist (see Bridger *et al.*, 1968).

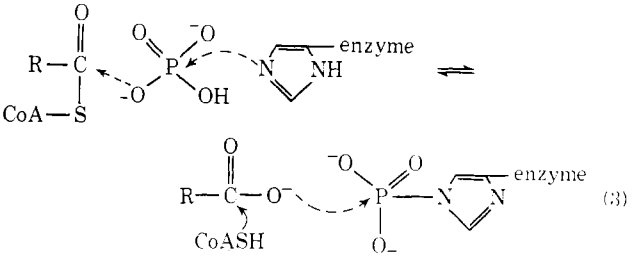
Two possibilities might rationalize the apparent discrepancy with specificity for succinate. The apparent K_m could be considerably less than the dissociation constant and the dephosphorylation reaction could depend upon the equilibrium amount of bound succinate. Secondly, the K_m, and possibly the K_d for succinate, increases as CoA concentration decreases, and could reach a value consistent with the concentrations required for dephosphorylation.

The observed rate of the dephosphorylation (Figure 3) and the lack of reversibility (Figure 4) rule out the succinate-dependent dephosphorylation as a catalytic step independent of presence of other substrates. Were this the case, the initial dephosphorylation should occur at a rate at least as great as the net catalytic rate with excess substrates. In contrast, the initial dephosphorylation rate with 91 mM succinate is only about $^1/_{10,000}$ of the potential net over-all rate. The ability of high succinate concentration to completely dephosphorylate E-^{32}P (Figure 3) suggests that some type of equilibrium phenomenon is involved. The lack of regeneration of E-^{32}P upon dilution (Figure 4) gives a strong argument against the results being due to presence of an enzyme-bound succinyl phosphate. The anticipated steps in such a case would be as depicted by eq 2, and removal of succinate by dilution should clearly result in regeneration of E-^{32}P. During net catalysis, no succinyl-P has been found in the reaction medium (Kaufman, 1955; Nishimura, 1967). Conceivably CoA could prevent such dissociation, and thus explain lack of detection of enzyme-bound succinyl-P, or CoA could react with E-succinyl-P more rapidly than succinyl-P dissociates.

$$\text{E-}^{32}\text{P} \xleftarrow{\text{succinate}} \text{E-P-succinate} \longleftrightarrow \text{E·succinyl-P} \qquad (2)$$

Although our results make it unlikely that formation of an enzyme-bound succinyl-P from E-P and succinate represents an independent step in the catalysis, the possibility must be considered that in the presence of other substrates the formation of the bound succinyl phosphate is greatly accelerated. Indeed, substrate synergism has been observed with the heart enzyme (Cha *et al.*, 1967), and studied in more detail with the *E. coli* enzyme as measured by the ability of CoA to accelerate the ADP → ATP exchange involving E-P (Bridger *et al.*, 1968). However, the acceleration observed was about 30-fold, as contrasted to the roughly 10,000-fold acceleration that would be required for the formation of enzyme-bound succinyl-P to be a catalytic intermediate. Such a marked effect means that other catalytic routes must continue to be given consideration.

An attractive possibility not involving either succinyl-P or an enzyme-CoA as an intermediate is that the reversible formation of E-P from P_i and succinyl-CoA occurs by a concerted mechanism, as depicted by eq 3. A site with catalytic capacity



(3)

for aligning substrates for such a concerted reaction might readily show slow reactions such as the dephosphorylation of E-P by succinate and the slow hydrolysis of ATP by the *E. coli* enzyme that does not appear to involve E-P formation (Ramaley *et al.*, 1967), or the similar GTP hydrolysis by the mammalian enzyme (Cha *et al.*, 1967). A concerted mechanism is obviously difficult to test by present techniques, but gains support if various experiments fail to demonstrate the participation of plausible intermediates.

The substrate specificity studies deserve further comment. The inability of oxy-CoA and the ability of 4-phosphopantetheine to promote the E-P $\rightleftarrows$ P_i exchange points to the prime importance of the SH group in the catalysis. The SH could be of unusual importance in the binding of CoA to the enzyme as indicated by the poor ability of oxy-CoA to inhibit net catalysis. Interaction of the SH with the enzyme could promote conformational change conducive to binding of the CoA molecules.

In previous tests with *E. coli* synthetase, Gibson *et al.* (1967) did not note any formation of malatyl-CoA. This likely reflects the substrate concentration used (1 mM malate) and the ability of their assay to detect rates about 1% of that observed with succinate as a substrate. The E-P dephosphorylation test readily detects malate participation. In addition, we have observed that at a 1 mM concentration malate is only about 0.1% as effective as succinate, while at 91 mM the rate with malate is 3% of that with succinate. This difference likely reflects a higher K_m for malate. With the mammalian synthetase, a procedure based on the stimulation of the E-P $\rightleftarrows$ P_i exchange suggests that fumarate and α-ketoglutarate but not malate would partially replace succinate (Butler, 1964). The organic acid specificity of the synthetase appears to show species variation.

The possibility that the observed reactions with malate result from presence of succinate in the malate appears unlikely. The extent of dephosphorylation of E-P by succinate requires high succinate concentration, and 91 mM malate dephosphorylates nearly to the same extent as 91 mM succinate (Table II). Also, the rate of dephosphorylation observed with malate is about one-fifth of the rate with succinate, but in the over-all catalysis, malate is only 3% as effective as succinate.

Added in Proof

Since this paper was submitted, reports have appeared that desulfo-CoA will accelerate succinyl phosphate synthesis (Grinnel and Nishimura, 1969) and formation of ATP from succinyl phosphate (Hildebrand and Spector, 1969) by succinyl-CoA synthetase. If in the presence of desulfo-CoA the rate of E–P dephosphorylation by succinate is equal to the potential net catalytic rate, the possible role of succinyl phosphate as an intermediate would be strengthened considerably.

References

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