A Phosphorus 31 Nuclear Magnetic Resonance Study of the Intermediates of the Escherichia coli Succinyl Coenzyme A Synthetase Reaction

EVIDENCE FOR SUBSTRATE SYNERGISM AND CATALYTIC COOPERATIVITY*

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Escherichia coli succinyl-CoA synthetase has an αβ2 structure (molecular weight, 140,000) with the two active sites arranged at the interface of the α and β subunits. Here we describe 31P-NMR experiments confirming the existence of two phosphorylated intermediates in catalysis. The phosphohistidyl resonance is readily observed at -4.8 ppm. This resonance is shifted upfield in the presence of Mg2+ and broadened by Mn2+, indicating interaction between the metal ion and the phosphoryl group. The addition of succinate alone is without effect on the spectrum. The presence of CoA causes a downfield shift and broadening of the phosphohistidyl resonance to a line width sufficiently large to be indicative of two exchanging conformations. The presence of both CoA and the competitive inhibitor 2,2'-difluoro-d-succinate seems to freeze the phosphoryl group in one orientation. The addition of ATP to this already phosphorylated enzyme leads to appearance of a succinyl-phosphate resonance. This last result is in harmony with the concept of alternating sites catalytic cooperativity previously proposed for this enzyme, since it implies that ATP binding or phosphorylation of histidine at one active site triggers phosphoryl transfer from histidine to succinate at the other site. If the nonhydrolyzable βγ-methylene analogue of ATP is used, however, this produces no change in the phosphohistidyl resonance. This suggests that intrasubunit communication is triggered by phosphorylation by ATP rather than by binding of nucleotide. We also observed CoA-induced changes in the spectrum of the enzyme-bound βγ-methylene analogue of ATP, consistent with the previously observed CoA-mediated enhancement of the ATP → ADP exchange which is known as "substrate synergism." A detailed model for catalysis consistent with these observations is presented.

Sucinyl-CoA synthetase from E. coli is a tetrameric enzyme with an αβ2 structure and an overall molecular weight of 140,000 (1, 2). The enzyme is best described as a dimer of dimers, since it has been shown that it contains two active sites, each arranged at the interface of an α and a β subunit (3-5). The reaction catalyzed is believed to proceed via three partial reactions with a covalent N-3 His-P' residue and an enzyme-bound Suc-P (6) participating as discrete intermediates.

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E + ATP = E·P + ADP
\]

(1)

\[
E·P + succinate = E·Suc-P
\]

(2)

\[
E·Suc-P + CoA = E + succinyl-CoA + P
\]

(3)

\[
\text{Sum: ATP + succinate + CoA = succinyl-CoA + ADP + P}
\]

(4)

It has been shown that the phosphorylated enzyme satisfies the kinetic criteria required of an obligatory intermediate in catalysis (7). The catalytic competence of the Suc-P intermediate (8) has been a subject of some controversy (9), which was settled principally by the demonstration that the rates of Suc-P utilization and production were comparable to the overall rates in the presence of the analogue desulfu-CoA (10, 11). This observation is one of the many demonstrations of a property of the enzyme known as substrate synergism, viz. the enzyme is fully active only when all substrate binding sites are occupied (7).

Another property of the enzyme that is pertinent to the subject of this paper is the apparent half-of-the-sites phosphorylation by ATP; only one of the two α subunits is readily phosphorylated (12, 13). Similar effects have recently been reported for malate thiokinase of Pseudomonas MA, which has a similar subunit structure and catalyzes a virtually identical reaction (14, 15). Since enzymes showing negative cooperativity or half-of-the-sites reactivity are likely candidates for alternating sites cooperativity, Bild et al. (16) investigated the enzyme for this property. They demonstrated an ATP modulation of the extent of oxygen exchange between medium [18O]P and [18O]succinate for the tetrameric E. coli enzyme. No modulation was detected for the dimeric pig heart enzyme, leading them to propose that the binding of ATP to one active site promotes catalytic events at the other (i.e. catalytic cooperativity) and that the two active sites work in an alternating fashion (i.e. alternating sites cooperativity). Capacity for alternating sites cooperativity has been confirmed for the E. coli enzyme (17).

In this paper, we report 31P-NMR studies of the two catalytic intermediates. While 31P-NMR has provided useful information about catalytic involvement of the active site phos-

* The abbreviations used are: His-P, phosphohistidine; Suc-P, succinyl-phosphate; AMPPCP, adenylyl-(β,γ-methylene)-diphosphonate.

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phoserine residue of E. coli alkaline phosphatase (18, 19) and the pyridoxal phosphate residue of rabbit muscle glycogen phosphorylase (20, 21), NMR studies on His-P-containing proteins to date have been primarily confined to determination of the position of phosphorylation of the histidyl residue (22-24). Since the enzyme has the property of substrate synergism and possibly that of alternating sites cooperativity, we have concentrated on the effects of the addition of substrates on the $^{31}P$-resonances observed for the N-3-His-P and Suc-P, respectively. Further evidence for substrate synergism and catalytic cooperativity has been obtained.

EXPERIMENTAL PROCEDURES

Succinyl-CoA synthetase was purified from Crooke’s strain of E. coli, grown on succinate-based medium, by methods described earlier (25, 26). Preparations were judged to be pure by homogeneity upon gel electrophoresis. Assays for activity were performed as described by Ramaley et al. (12). The concentration of the enzyme was determined from its absorbance at 280 nm (2). Samples for NMR experiments containing 1.0 phosphoryl groups/αβ tetramer were prepared as described earlier (27-29) except that Millipore molecular separators were used instead of ultrafiltration to concentrate the samples. It has been shown that this procedure efficiently eliminates paramagnetic impurities (28, 29). The specific activity was measured before and after obtaining $^{31}P$-NMR spectra and was never below 5 units/mg of enzyme. All NMR samples were in a buffer containing 40 mM Tris-HCl, 80 mM KCl, 25 mM D_2O, pH 7.2.

The enzyme A and adenosine 3’-diphosphate were obtained from P-L Biochemicals. ATP was from Terochem (Edmonton, Canada). 2,2-Difluorosuccinate was purchased from Sigma, Tris (ultra pure) from Schwarz-Mann, and D_2O from Biorad. Other chemicals were analytical grade. Phosphohistidine was synthesized as described by Rosenberg (30). SUC-P was prepared by mixing succinic anhydride (in ethanol) in a phosphate buffer (31). The $^{31}P$-NMR chemical shifts measured for these preparations are comparable to those seen for acethylphosphate (Sigma). $^{31}P$-NMR spectra were obtained as described earlier (32, 33) at a frequency of 100.3 MHz. The pulse angle was 90 °C; the acquisition time was 0.4 s and the recycle time was 2.0 s. Proton decoupling was used with all samples containing nucleotides, but decoupling had no significant effect on the resonance of the enzyme-bound His-P residue. Samples (1.5 ml) were placed in 10-mm precision tubes (Wilmad) equipped with Teflon vortex plugs. The sample temperature was kept constant at 27 °C. All spectra are plotted with 25 Hz additional line width by means of computer digital filtering, unless otherwise indicated.

RESULTS

Effects of Succinate and Mg$^{2+}$ on His-P Residue—Since the γ- and β-phosphoryl residues of ATP and ADP, respectively, in the presence of Mg$^{2+}$ give rise to a resonance in the $^{31}P$-NMR spectrum with similar chemical shift to those of His-P standards (22), we could not study phosphorylation of the enzyme (partial reaction 1) directly. Therefore, all subsequent experiments were performed with enzyme that was phosphorylated with ATP and isolated by gel filtration, according to the procedure of Wang et al. (27). The product is a preparation containing 1 phosphoryl group/αβ tetramer.

A typical $^{31}P$-NMR spectrum of E-P is shown in Fig. 1A. The chemical shift position (−4.8 ppm) is characteristic of an N-3 His-P residue (22). The frequency dependence of the line width, reported elsewhere (32), has led us to conclude that the residue is rigidly held to the enzyme and that it is in a monoanion form.

Figure 1B shows the spectrum of the same sample after addition of 5 mM succinate. Clearly, no changes in line width or chemical shift position occur. Even with concentrations of succinate up to 25 mM, only minor upfield shifts (<0.2 ppm) were observed. Although the succinate-binding site should be saturated under these conditions, the His-P seems hardly affected by its presence. The requirement for a metal ion for the partial reactions can be fulfilled by either Mg$^{2+}$ or Mn$^{2+}$ (35). Proton relaxation rate/enhancement studies have revealed four Mn$^{2+}$ binding sites with similar affinity for the phosphorylated and dephosphorylated enzyme (28, 29). Thus, it is not clear whether the phosphoryl group is directly involved in the binding of the metal ion. Fig. 1C shows that addition of 2.5 mM MgCl_2 leads to an upfield shift of 0.6 ppm (−4.8 to −5.4 ppm) as indicated by the arrows. Titration studies with Mg$^{2+}$ showed an increase in the His-P chemical shift with concentration and that high Mg$^{2+}$ concentration induces loss of the phosphoryl moiety. Reciprocal plots of change in chemical shift versus [Mg$^{2+}$] suggested a $K_M$ near 4 mM. This value agrees reasonably with the value of 10 mM obtained (35) for maximal activity of the enzyme. Since lower concentrations of Mn$^{2+}$ are necessary for maximal activity, this presumably also corresponds to the $K_M$ of 0.7 mM determined for Mn$^{2+}$ binding to the phosphorylated enzyme (28). The results of the Mg$^{2+}$ titrations were similar in the presence and absence of succinate. Since Mg$^{2+}$ effects on the His-P residue of the enzyme are
not sufficient sole evidence for direct interaction between the phosphoryl group and the metal ion, we studied the effect of addition of permanganate $\text{Mn}^{2+}$ ions. This caused broadening of the residue beyond detection, which was reversible by the addition of EDTA. This places the metal ion within 10 Å of the phosphoryl group, suggesting that it interacts with it but is not necessarily coordinated to it. Therefore, we propose that the binding site for the metal ion is mainly supplied by the monooxidonic His-P residue. Such a mode of binding would explain the small difference in the $K_d$ values found for $\text{Mn}^{2+}$ binding to phosphorylated and dephosphorylated enzyme. The line widths of the resonances observed for these residues are indicative of binding of the coenzyme to the enzyme, since they exceed those measured for a sample of CoA in the absence of enzyme. The low $K_d$ for CoA (1.5 μM) (1) would suggest slow exchange between free and bound coenzyme. However, no separate peaks have been observed; perhaps the chemical shifts for the bound and free form are identical. Unfortunately, several trials have not allowed clear definition of the mode of binding. The binding of CoA is very sensitive towards small changes in the specific activity of the enzyme (34), and this was further substantiated by our spectral data. Fig. 2C shows clearly that partial reaction 2 can occur in the presence of CoA. Since no Suc-P line is observed, one might infer that Suc-P is not a true intermediate and that partial reactions 2 and 3 occur in concerted fashion. However, evidence is presented below that supports the intermediate formation of Suc-P, indicating that partial reactions 2 and 3 are both fast, relative to the time course of the experiment. Clearly, this is another demonstration of substrate synergism, namely that the presence of CoA enhances the rate of partial reaction 2. This is similar to the earlier demonstration of the effect of the analogue desulfo-CoA on the rate of Suc-P production and utilization (10, 11).

The means by which CoA stimulates partial reaction 2 is further examined by the series of spectra shown in Figs. 3 and 4. Comparison of Fig. 3, A and B shows that CoA addition results in a downfield shift from $-4.8$ to $-4.1$ ppm, together with broadening of the 55 Hz-wide His-P resonance. In a separate paper (32), we have reported the frequency dependence of the line width for the His-P residue. The His-P residue is immobilized on the protein surface, and thus the observed line width of 55 Hz is an upper limit. Resonances with line widths exceeding this figure, as we see here for the His-P residue after addition of CoA, must, therefore, originate from exchange broadening, and are taken as evidence for the existence of not less than two exchanging conformations (32). The other resonances in spectrum B of Fig. 3 originate from CoA ($-10.1$ and $3.7$ ppm) and from P at $2.1$ ppm. The appearance of P, here consistent with the report (11) that CoA can induce an ATPase activity in the enzyme. Apparently, the His-P is most stable in its conformation signalled by a chemical shift of $-4.8$ ppm. Both the $\text{Mg}^{2+}$-induced upfield shift and the CoA-induced broadening and downfield shift lead to loss of enzyme His-P to solution inorganic phosphate.

A titration study of the broadening effect, this time in the presence of $\text{Mg}^{2+}$ and 2.5 mM dithiothreitol, showed that at half saturation (1 CoA/tetrameric enzyme molecule, Fig. 4C) the effect is largely manifested as a downfield shift ($-5.4$ to $-5.1$ ppm) with almost a doubling of the line width to 100 Hz. (These shifts are higher than the usual $-4.8$ ppm because of the presence of 2.5 mM MgCl₂ (see Fig. 4, A and B). It is noteworthy that the CoA-induced downfield shifts and the

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Other possible explanations for the broadening that accompanies CoA addition include an increase in the chemical shift anisotropy factor for the enzyme-bound His-P residue (as would be expected for a CoA-induced distortion of the phosphoryl group to facilitate nucleophilic attack by succinate), or an increased rotational correlation time that might be attributable to a CoA-induced aggregation of the enzyme. However, previous studies using purified subunits (3) indicated exchange of the His-P residue between two environments, and our data seem to support this concept.
NMR Studies of Succinyl-CoA Synthetase

Fig. 3. $^{31}$P-NMR spectra of 19.0 mg/ml of succinyl-CoA synthetase with the following additions. A, no additions, 27,500 scans; B, 2 mM dithiothreitol, 0.50 mM CoA, 20,000 scans (dithiothreitol alone has no effect on spectrum); C, as in B, plus 5 mM 2,2'-difluorosuccinate, 20,000 scans. Other conditions as described under "Experimental Procedures." For assignment of peaks see text and Fig. 2. Mg$^{2+}$-induced upfield shifts are additive.) Subsequent saturation of the total enzyme molecule shows a further downfield shift to $-4.5$ ppm and more severe broadening (see Fig. 4D). Without knowledge of the order of CoA binding to the two active sites (of which one is phosphorylated) it is not possible to distinguish if these effects are caused by binding to the phosphorylated or to the other active site. Since saturation of the enzyme is required for the full effect on the His-P, it seems that binding to both active sites is a necessity for the observed changes.

The addition of CoA in either the presence or absence of Mg$^{2+}$ gave rise to extensive broadening and downfield shifts in six separate experiments but no consistent line shape could be found (compare Figs. 3B, 4D, and 5C). It should be borne in mind that the previously mentioned differences in CoA binding and the CoA-induced ATPase activity complicate such measurements. Similar problems have been encountered with other enzymes.

In a subsequent experiment, instead of adding succinate and MgCl$_2$ (which would lead to the result in Fig. 2C), we added the competitive inhibitor 2,2'-difluorosuccinate (Fig. 3C). The broadened resonance clearly sharpens to a resonance with a line width indicative of only one conformation (32), and shifts back upfield to $-4.65$ ppm. Apparently, the phosphoryl group is now locked into one position, perhaps ready for transfer if the correct substrate (succinate) were in place. Although the chemical shift position of this resonance is virtually indistinguishable from that observed for phosphorylated enzyme in the absence of substrates, this does not necessarily imply the same conformation.

When the additions in the experiment of Fig. 3 were made in the opposite order (by adding fluorosuccinate first and CoA second) the identical end result was obtained, indicating that the order of addition is not important.

Because of the effects of desulfo-CoA on Suc-P production and utilization (10, 11), it was of interest to determine whether CoA analogues could give a similar broadening, since this would directly relate the observed broadening to the enhancement of catalytic events. The presence of 1 mM adenosine 3',5'-diphosphate (i.e. CoA without the pantothenoic acid residue) did not cause any broadening or change in chemical shift. Consistent with this is that no effect on the rate of Suc-P production could be demonstrated (data not shown). Similarly the analogue oxy-CoA did not exert effects on this partial reaction (10). Thus, these observations are consistent with the

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Fig. 4. $^{31}$P-NMR spectra of 26 mg/ml of succinyl-CoA synthetase with the following additions. A, no additions, 7,000 scans; B, 2.5 mM MgCl$_2$ plus 2 mM dithiothreitol, 12,500 scans; C, as in B, plus 0.12 mM CoA, 10,000 scans; D, as in C, but CoA concentration increased to 0.60 mM, 8,500 scans. Other conditions are described under "Experimental Procedures." The peak assignments are similar to those of Figs. 2 and 3.
P-NMR Studies of Succinyl-CoA Synthetase

proposal that the CoA-induced broadening signals the presence of two exchanging conformations for the His-P, and that exchange between these two conformations is necessary for catalysis.

Effects of ATP on Phosphohistidine Residue—The concept of catalytic cooperativity would imply that ATP binding to one active site promotes catalytic events at the other (16). The binding of Mg-ATP to phosphorylated enzyme is known to be very weak (28, 34). Fig. 5 shows again that partial reaction 2 does not occur independently (Fig. 5, A and B). When ATP is added (Fig. 5C), it is clear that the His-P resonance is obscured by the ATPγ (−4.9 ppm) and ADPβ (−5.6 ppm) resonances. However, two new resonances appear in the downfield region; the one at 2.2 ppm is P, and the one at −1.5 ppm we have assigned to Suc-P. The narrowness of the Suc-P line indicates that if this compound is bound to the enzyme it is not rigidly held. It is also possible that this noncovalent intermediate is released from the enzyme; since it is not very stable in solution (31), it would hydrolyze to succinate and P. This would account for the appearance of the P, resonance in the spectrum. In another experiment where the time course of this reaction was followed, the ATP resonance declined, the Suc-P passed through a maximum, and only the P, and ADP resonances kept increasing (data not shown) in keeping with the idea that Suc-P is released from the enzyme and subsequently hydrolyzed in solution. Since these data indicate that the affinity of the enzyme for this intermediate is low, it is remarkable that during normal catalysis this intermediate is not detectable in solution. Apparently, partial reaction 3 must be fast, consistent with our interpretation of Fig. 2C. In fact, it has been demonstrated that it proceeds non-enzymatically at a considerable rate in solution (37).

Effects of Binding of AMPPCP—The results presented in Fig. 5 give strong support to the proposal that ATP binding to one active site (nonphosphorylated) triggers catalytic events at the other site (phosphorylated) (16). Since use of ATP does not allow differentiation between effects caused by binding or phosphorylation by the nucleotide, we approached this question by the use of nonhydrolyzable ATP analogues. The Mg2+ complex of β,γ-imido-ATP could not be used since its β resonance would overlap that of His-P. The β,γ-methylene ATP analogue is a competitive inhibitor (K, 0.38 mM), has more advantageous chemical shifts, and the presence of Mg2+ bears close resemblance to ATP (33). Fig. 6, A and B clearly indicate that the presence of saturating amounts of this analogue does not bring about changes in the phosphohistidyl residue. The peaks at 15.0, 9.9, and −10.3 ppm are assigned, respectively, to the γ-, β-, and α-phosphorus atoms of the analogue. Upon addition of Mg2+ (Fig. 6C), large changes in the analogue spectrum are observed (33), but the His-P shows its normal Mg2+-induced upfield shift (−4.8 to

Fig. 5. 31P-NMR spectra of 22 mg/ml of succinyl-CoA synthetase with the following additions. A, no additions, 27,500 scans; B, 5 mM succinate and 4 mM ADP, 12,500 scans; C, as in B, plus 2.5 mM ATP, 2,500 scans (linebroadening 10 Hz). Other conditions are as described under "Experimental Procedures."
Effects of CoA on AMPPCP Binding—To further substantiate that binding of the ATP analogue AMPPCP does not lead to changes in the properties of the enzyme, we performed the experiments depicted in Fig. 7. The results of Fig. 7, A and B are consistent with an earlier observation (Fig. 6) that binding of AMPPCP or Mg-AMPPCP does not lead to any changes in the chemical shift position or line width of His-P (~4.8 ppm). The peaks in spectrum A of Fig. 7, going from downfield to upfield, are assigned to the y, β, and α of the analogue, respectively. Addition of MgCl₂ causes expected changes in the AMPPCP spectrum. Note that the His-P has undergone its characteristic Mg²⁺-induced upfield shift (~4.8 to ~5.5 ppm). Addition of CoA (spectrum C of Fig. 7) leads to the characteristic downfield shift (to ~4.3 ppm) and broadening of the phosphohistidyl residue. In spectra C and D of Fig. 7 the resonances at 3.8 and ~10.6 ppm (overlapping with AMPPCPγ) originate from CoA. Subsequent addition of 2,2'-difluorosuccinate leads to an upfield shifted sharp resonance (~5.1 ppm) as seen earlier in the absence of the ATP-analogue. Thus, the presence of AMPPCP does not lead to changes in the measurable properties of the enzyme, in keeping with the observation that it does not induce Suc-P production (Fig. 6).

Clearly, the β- and γ-P resonances (the two most downfield peaks) of AMPPCP are affected by the addition of CoA (compare Fig. 7, B and C). This is further substantiated by the data of Table I. Comparison of the chemical shifts measured for the ATP analogue in the presence and absence of enzyme, but in the absence of CoA, shows that only small changes occur upon binding to the enzyme. Upon addition of CoA (in the presence and absence of 2,2'-difluorosuccinate) a change in the β resonance of 0.3 ppm is observed. Assuming that fast exchange conditions prevail we can apply the formula δobs = δfree−δfree + δbound−δbound, with δf and δb, respectively, the chemical shift and mole fraction. Since the enzyme is phosphorylated at one active site, it presumably has only one binding site for AMPPCP; therefore, we calculate for the bound form that the β resonance is shifted downfield by 7.5 ppm and the γ resonance is shifted upfield by 1.7 ppm. (If one assumes two binding sites for ATP, the shifts are 3.7 and 0.8 ppm, respectively.) The chemical shifts calculated in this way clearly place the enzyme-bound form outside of the range of chemical shifts measured for solution structures (33). The directions of the shifts suggest that the bound form is in the deprotonated form with Mg²⁺ ion tightly complexed especially to the β phosphorous of the ATP analogue. This “tightening up” of the AMPPCP binding by CoA may be the basis for the earlier observed enhancement of the ADP → ATP exchange reaction by CoA (7) which was the first demonstration of the substrate synergism for this enzyme.

The change in chemical shift calculated for the bound form of the ATP analogue (7.5 ppm) is large compared to those reported earlier for ATP binding to other kinases (36) and may be a reflection of the greater sensitivity of chemical shifts of methylene compounds towards changes in environment (33).

DISCUSSION

The spectra presented here show for the first time that the His-P resonance on a protein of this size can be readily observed. The chemical shift of the phosphohistidyl residue is very close to that of His-P in solution (22). In the case of alkaline phosphatase (18, 19), a large downfield shift was reported for the active site phosphoserine residue which was attributed to bond strain. Similarly, studies on the bacterial heat-stable phosphoryl carrier protein (HPγ) showed a downfield shift for the N-1-phosphohistidine (22, 23), whereas the resonance for one of the N-3-phosphohistidines of histone displays a large upfield shift (24). Our data, together with the normal shift of the phosphoserine residue of phosphoglucomutase (38), show that a displacement of chemical shift should not necessarily be anticipated for active site phosphorylated amino acid residues.

Only a sharp resonance was seen for Suc-P, suggesting either a low affinity of the enzyme for this intermediate, or a high degree of flexibility of the phosphoryl group of enzyme-bound Suc-P. Such flexibility might be expected for acyl the β and γ resonances. This differs from Fig. 5C because of a change in the Mg²⁺/nucleotide ratio. Full titration curves are described elsewhere (33).
phosphate intermediates, in view of the implied rotational freedom of the phosphoryl group of glutamylphosphate during catalysis by glutamine synthetase (39).

Our studies have provided insight into the role of the Mg$^{2+}$ ion required for maximal enzyme activity. First, the Mg$^{2+}$ ion seems to be tightly complexed to the $\beta$-phosphoryl group of the enzyme-bound AMPPCP (Fig. 7), perhaps a necessity for phosphoryl transfer from ATP to the histidine since phosphorylation by Mg-ATP occurs at a rate far exceeding that observed with ATP (13). Secondly, the Mg$^{2+}$ ion seems to be close enough to the His-P residue to be involved in the phosphoryl transfer from histidine to succinate (Fig. 1). A second sphere metal ion complexation was suggested for the active site phosphoserine residue of phosphoglucomutase (38), and recent $^{31}$P-NMR experiments of alkaline phosphatase have shown direct coordination of $^{13}$Cd to P, but not to the phosphoserine residue (40). All of these considerations support the idea of a direct role for the metal ions in phosphoryl transfer.

Addition of only succinate or its competitive inhibitor 2,2'-difluorosuccinate has no effect on the chemical shift of the His-P resonance (Fig. 1). The presence of CoA causes a downfield shift in the His-P line that is additive with the Mg$^{2+}$-induced upfield shift. The CoA-induced broadening (Figs. 3B, 4D, and 7C) can be most easily understood in terms of the scheme shown in Fig. 8. The His-P residue is on the smaller $\alpha$ subunit and the succinate and CoA binding sites are on the $\beta$ subunit (3-5). Binding of CoA is shown to permit at least two exchanging conformations for the His-P. One may allow for dephosphorylation by ADP and the other for transfer to the succinate residue to form Suc-P. Such a mechanism is also consistent with results obtained with separated subunits (3) and with CoA-induced mobility as inferred from dansyl fluorescent label studies. The presence of both succinate and CoA freezes the residue in one position ready for transfer (see Figs. 3C and 7D). This agrees with the proton relaxation rate enhancement studies (28) that indicated a closed active site structure only in the presence of all substrates.

Our $^{31}$P-NMR data showing that phosphorylation by ATP promotes Suc-P production (see Figs. 5 and 6) can best be explained in terms of a catalytic cooperative model (41, 42). Since both active sites are capable of catalysis (17), we favor an alternating site model over an asymmetric one-sided cooperative mechanism such as that recently observed for metal-deficient alkaline phosphatase (43). Our model is detailed in Fig. 9. The arrangement of the subunits is chosen according to cross-linking data (44); also the order of addition of substrates is consistent with the reported kinetic mechanism, which indicated binding of ATP first, followed by random

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**Fig. 8.** Schematic representation of the active site of succinyl-CoA synthetase at the point of contact between the two subunit types. In this representation, the His-P residue is visualized as being sufficiently mobile due to the binding of CoA to allow phosphoryl transfer either to ADP on the $\alpha$ subunit (left) or to succinate on the $\beta$ subunit (right). See text for further explanation.

**Fig. 9.** Proposed model for alternating site catalytic cooperativity for *E. coli* succinyl-CoA synthetase. Different conformations for the two $\alpha\beta$ halves of the molecule are indicated by hatching and dots. Reciprocal conformational change is shown to be promoted by phosphorylation of one active site, accompanied by accelerated transfer of the phosphoryl group from the His-P to succinate at the other active site. See text for further explanation.
addition of CoA and succinate (45). The dephosphorylated apoenzyme (left of Fig. 9) is clearly in a different conformation as indicated by its greatly enhanced proteolytic susceptibility (13). Phosphorylation results in a different conformation characterized by two unequivocal active sites. The exchanging conformations induced by CoA and the subsequent linking by succinate are symbolized in the next set of diagrams of Fig. 9 (see also Figs. 3 and 8); the end result is not dependent on the order of the addition. This arrangement of substrates could imply that the step in the reaction that is most sensitive to catalytic cooperativity is the transfer of phosphoryl from His-P to succinate. This is in contrast to the earlier proposals that substrate synergism is not necessarily mediated within one active site but could also be another manifestation of catalytic cooperativity between the two active sites.

Since CoA affects the binding of ATP analogues (Fig. 7, Table I), and phosphorylation by ATP stimulates Suc-P production on the opposite active site, attachment of CoA to both β subunits may be a prerequisite for optimal catalytic activity. Similarly, the CoA-induced broadening effect (Figs. 3 and 4) becomes maximal only when both binding sites for this nucleotide are occupied. These considerations suggest that the originally observed CoA-induced enhancement of partial reactions termed substrate synergism is not necessarily mediated within one active site but could also be another manifestation of catalytic cooperativity between the two active sites. CoA-induced stimulation of GTP → GDP exchange in the dimeric pig heart enzyme (47) is not easily explained in this way, however.

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REFERENCES

Table I

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31P-NMR Studies of Succinyl-CoA Synthetase
A phosphorus 31 nuclear magnetic resonance study of the intermediates of the Escherichia coli succinyl coenzyme A synthetase reaction. Evidence for substrate synergism and catalytic cooperativity.

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