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Novel Mechanisms of *Escherichia coli* Succinyl-Coenzyme A Synthetase Regulation

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Low concentrations of ADP are shown to increase the rate of phosphoenzyme formation of *E. coli* succinyl-coenzyme A (CoA) synthetase (SCS) without altering the fraction of phosphorylated enzyme. This is true when either ATP or succinyl-CoA and P_i are used to phosphorylate the enzyme. The stimulatory effect of ADP is not altered by sample dilution, is retained upon partial purification of the enzyme, and reflects the binding of ADP to a site other than the catalytic site. GDP also alters the phosphorylation of the *E. coli* SCS but does so primarily by enhancing the level of the phosphoenzyme and only when ATP is used as the phosphate donor. GDP appears to function by neutralizing the action of a specific inhibitory protein. This inhibitor of SCS allows for interconversion of succinate and succinyl-CoA in a manner dissociated from changes in ATP-ADP metabolism. These previously unidentified and varied mechanisms by which SCS is regulated focus attention on this enzyme as an important control point in determining the cell's potential to meet its metabolic demands.

The processes of cell differentiation, growth, and aging reflect the selection of specific pathways to be activated or inactivated. Such metabolic regulation may occur at the level of expression of components in those pathways or by modulating their activities. We have been investigating how changes in energy metabolism reflect or determine the cell's genetic potential for growth and development. In particular, our studies have focused on the regulation of succinyl-coenzyme A (CoA) synthetase (SCS), the only enzyme in the citric acid cycle that catalyzes a substrate-level phosphorylation reaction. This reaction is completely reversible, allowing for the production of nucleoside triphosphate (NTP) during aerobic metabolism and the synthesis of succinyl-CoA for anabolic reactions.

Partial reactions

$$NTP + E \leftrightarrows NDP + E{\sim}P$$

$$E{\sim}P + \text{succinate} \leftrightarrows E \cdot \text{succinate}{\sim}P$$

$$E \cdot \text{succinate}{\sim}P + CoA \leftrightarrows E + \text{succinyl-CoA} + P_i$$

Total reaction

$$NTP + \text{succinate} + CoA \leftrightarrows NDP + \text{succinyl-CoA} + P_i$$

where NDP is nucleoside diphosphate and E is the enzyme. As seen in the schema above, the phosphoenzyme is an intermediate in the reaction and can be formed by autophosphorylation with either an NTP (top partial reaction) or succinyl-CoA and P_i. The enzyme is composed of two distinct protein subunits, α and β. There are two known forms of the enzyme. In gram-negative bacteria such as *Escherichia coli*, the enzyme is a tetramer of two α and two β subunits. It preferentially uses adenine nucleotides and will be referred to here as the A-form enzyme. G-form enzymes, which appear to use exclusively guanine nucleotides in the reaction, predominate in eukaryotes and gram-positive bacteria. They function as dimers composed of one α and one β subunit. The physiological significance of the differences in quaternary structure and nucleotide preferences with respect to enzyme action or organization in the citric acid cycle is unknown. In either case, it is a histidine residue in the α subunit that is phosphorylated to form the phosphoenzyme intermediate. (for reviews, see references 5 and 14).

In the presence of saturating or near-saturating concentrations of substrates, SCSs from both *E. coli* and mammalian sources display an unusual kinetic behavior termed substrate synergism, whereby the presence of a substrate for one partial reaction stimulates another partial reaction (4). Since the enzyme in *E. coli* is a tetramer, substrate synergism has been explained by alternate-site cooperativity, where binding of a substrate to one catalytic site enhances the reactivity of the catalytic site of the other dimer (3). To explain substrate synergism in G-form enzymes, which are dimers, the same site model has been proposed (15). In this case, full occupancy of substrate binding sites in the same active site enhances the reactivity of the enzyme. To date, the mechanism(s) underlying substrate synergism has not been delineated.

Despite the attention SCS has received with respect to its enzyme mechanism, its role in the citric acid cycle has not been fully appreciated. The reversible nature of its reactions and the apparent absence of any higher-order regulation would suggest that SCS is not a critical control point for the flow of substrates through the cycle. However, recent experiments by Um and Klein (18–20) have provided evidence that the activity of G-form enzymes is allosterically regulated. At concentrations above 5×10^{-6} M, GDP binds to the catalytic site of the enzyme. As expected from the known enzyme mechanism, GDP occupancy of that site competitively inhibits phosphoenzyme formation by GTP and enhances the dephosphorylation of the phosphoenzyme. In contrast, low concentrations of GDP, between 1×10^{-8} and 2×10^{-6} M, increase the rate of phosphoenzyme formation when either P_i plus succinyl-CoA or GTP is used as the substrate. These and other data indicate that at low concentrations, GDP binds to a relatively high affinity allosteric site. The allosteric site for GDP can also be distinguished from the catalytic site by GDPβS, which is able to bind to the catalytic site but not to the allosteric site (8). In this

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paper, we show that this type of regulation is not specific to G-form enzymes but that A-form enzymes are similarly regulated but preferentially by ADP as opposed to GDP. We also report another level of regulation for the A-form enzyme. *E. coli* cells contain a protein that functions to limit the fraction of phosphoenzyme formed and does so only when NTP is used as a phosphate donor. The inhibitory action of the protein is relieved by the addition of high concentrations of GDP. The data indicate several novel and complex levels of regulation of SCS that would render the enzyme highly responsive to changes in the cell's metabolic demands.

MATERIALS AND METHODS

SCS preparations. *E. coli* W3110 cells were grown in succinate medium (8) and lysed by sonication as described by Ramaley et al. (17). Residual intact cells and cellular debris were removed by centrifugation for 10 min at 12,000 × *g*. The supernatant fraction was extensively dialyzed against buffer A (50 mM Tris-HCl [pH 8.0], 10 mM $MgCl_2$, 10% glycerol, plus a mixture of nine protease inhibitors (11) to remove any endogenous enzyme substrates and stored at −20°C. Where indicated, ammonium sulfate was added to the lysate to achieve a final concentration of 50%. Precipitated material was collected by centrifugation at 12,000 × *g* for 10 min. Pellets were resuspended in buffer A, and both supernatant and pellet fractions were then dialyzed against that buffer. The presence of protease inhibitors in the buffer was particularly necessary to maintain the activity of pellet fraction in reconstitution experiments when samples were stored for any period of time. For reconstitution experiments, various aliquots of the ammonium sulfate pellet fraction were incubated at room temperature with a fixed amount of the supernatant fraction. At the indicated times, the level of SCS phosphorylation was determined as described below except that the phosphorylation reaction was allowed to proceed for 15 min to ensure that the maximum amount of SCS had been phosphorylated.

Phosphorylation assays. Phosphorylation assays were performed at room temperature in buffer A and have been previously described in detail (18–20). Briefly, phosphorylation was initiated by incubating samples with either [γ-^{32}P]ATP (1.5 to 3.0 Ci/μmol; 1×10^{-8} to 3×10^{-8} M) or 1×10^{-8} M succinyl-CoA and 1×10^{-8} M $^{32}P_i$ (1 Ci/μmol) for the indicated times. These concentrations of substrates are optimal for demonstrating the NDP stimulation of the rate of radiolabeling of the G-form enzyme. These conditions do not support the dephosphorylation of the α subunit of SCS; thus, increases in the rate of SCS phosphorylation or fraction of SCS phosphorylated are not explained by an inhibition of enzyme dephosphorylation. Additionally, since no other phosphorylated proteins are detected by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and autoradiography under our assay conditions (see Fig. 4 and 5), any changes in SCS phosphorylation do not reflect phosphotransfer from another protein. At the indicated times after phosphorylation, radiolabeling of the α subunit of SCS was determined by autoradiography of SDS-polyacrylamide gels (12) that had been fixed for 30 min in 15% methanol. Acetic acid was not present during the fixation, since acid removed the radiolabel from SCS, a characteristic of histidine-phosphate (7). Relative intensities of radiolabeling were determined by using the Bio-Rad model 620 video densitometer and the 1-D Analyst software.

Other assays. Protein determinations were done according to the method of Peterson (16). Partial purification of SCS on hydroxyapatite was performed as previously described (2). The stability of the radiolabel in the reaction mixture was monitored by thin-layer chromatography on polyethyleneimine-cellulose MN 300 plates in 0.75 M KH_2PO_4, pH 3.4, as previously described (19).

Materials. [γ-^{32}P]ATP and $^{32}P_i$ were purchased from New England Nuclear, and [γ-^{32}P]GTP was from ICN. Other nucleotides were obtained from Pharmacia. All other chemicals were purchased from Sigma.

RESULTS

Effect of NDP on the phosphorylation of *E. coli* SCS. To investigate the regulation of A-form enzymes by NDP, we phosphorylated *E. coli* SCS with succinyl-CoA and $^{32}P_i$ in the absence or in the presence of NDP. As shown in Fig. 1A, ADP enhanced the apparent rate of phosphorylation of the α subunit of *E. coli* SCS, as reflected by the change in the initial slope of the time course of the phosphorylation reaction. Stimulation was detectable with 1×10^{-8} M ADP and increased with increasing concentrations of ADP up to 5×10^{-6} M (data not shown). This latter condition was optimal, producing a sevenfold increase in the apparent reaction rate without significantly altering the final level of phosphoenzyme formed. Higher concentrations of ADP resulted in lower levels of phosphoenzyme, a result expected when ADP binds to the catalytic site of the enzyme, allowing phosphotransfer to result in the formation of ATP (3, 4). GDP was also able to enhance the rate of phosphoenzyme formation but to a lesser extent than ADP. Maximal stimulation was seen with 5×10^{-6} M GDP, which increased the rate of phosphorylation only two- to threefold. In no case was SCS phosphorylation observed when succinyl-CoA was omitted, indicating that the stimulation does not reflect another metabolic process(es), e.g., the incorporation of $^{32}P_i$ into NDP to generate [γ-^{32}P]NTP.

When *E. coli* SCS was phosphorylated with [γ-^{32}P]ATP (Fig. 1B), the effects of ADP were similar to those seen when succinyl-CoA and $^{32}P_i$ were used as substrates. ADP (5×10^{-6} M) increased the initial rate of formation of the phosphoenzyme four- to eightfold while increasing the plateau of phosphorylation less than twofold (Fig. 1B, inset). Higher concentrations of ADP inhibited phosphorylation, a reflection of the binding of ADP to the catalytic site and the resulting competitive inhibition of ATP binding. Surprisingly, micromolar concentrations of GDP resulted in increased amounts of phosphoenzyme formed. SCS is phosphorylated on a His residue of the α subunit. The phosphate label incorporated into SCS in the presence of GDP was completely removed by acid treatment, a characteristic of P-His (7). Thus, the enhanced level of phosphoenzyme found is not expected to reflect its phosphorylation at a site other than the active-site His. The maximally effective concentration of GDP was 5×10^{-5} M, which normally led to a 10- to 15-fold increase in the amount of phosphorylated α subunit. These results are not explained by the binding of GDP to either the regulatory site (which stimulates the rate of phosphoenzyme formation) or the catalytic site, suggesting that an additional component determines the ability of SCS to use ATP as a substrate.

The action of GDP, but not ADP, is sensitive to sample dilution. We next assessed the premise that an additional factor(s) was necessary for the action of GDP on the phosphorylation of *E. coli* SCS by monitoring its effects at different dilutions of the sample preparation (Fig. 2). The ability of 5×10^{-5} M GDP to enhance the fraction of *E. coli* enzyme that was phosphorylated was strongly dependent upon protein concentration, such that the stimulation was greatly reduced when the sample was diluted 10-fold (Fig. 2B). A 100-fold dilution completely abolished the stimulation (Fig. 2C). The inability of this concentration of GDP to enhance the fraction of SCS phosphorylated in diluted samples was not due to the inactivation of either the enzyme or the other required component. The effect was fully recovered upon reconcentration of the reaction mixture, using a Centricon 10 microconcentrator. Since this technique eliminates low-molecular-weight molecules, these results indicate that the additional component is a protein larger than 10 kDa and that the ability of GDP to increase the amount of SCS phosphoenzyme reflects some type of protein-protein interaction. These results dramatically contrast those obtained when the effects of ADP on the rate of SCS phosphorylation were examined. ADP (5×10^{-6} M) decreased the time required to achieve maximum phosphorylation under all the dilutions tested. Although the effect of ADP on the rate of the reaction was more dramatic when times earlier than 1 min were examined, it was increasingly difficult to make such early measurements in the more dilute samples. The 1-min time points permitted accurate determinations under all conditions. The data indicate that the effect of this concentration of ADP, like that of low concentrations of GDP on G-form enzymes, reflects a direct action on the enzyme. This was further substantiated by the finding that ADP was equally effective in stimulating the rate of phosphoenzyme

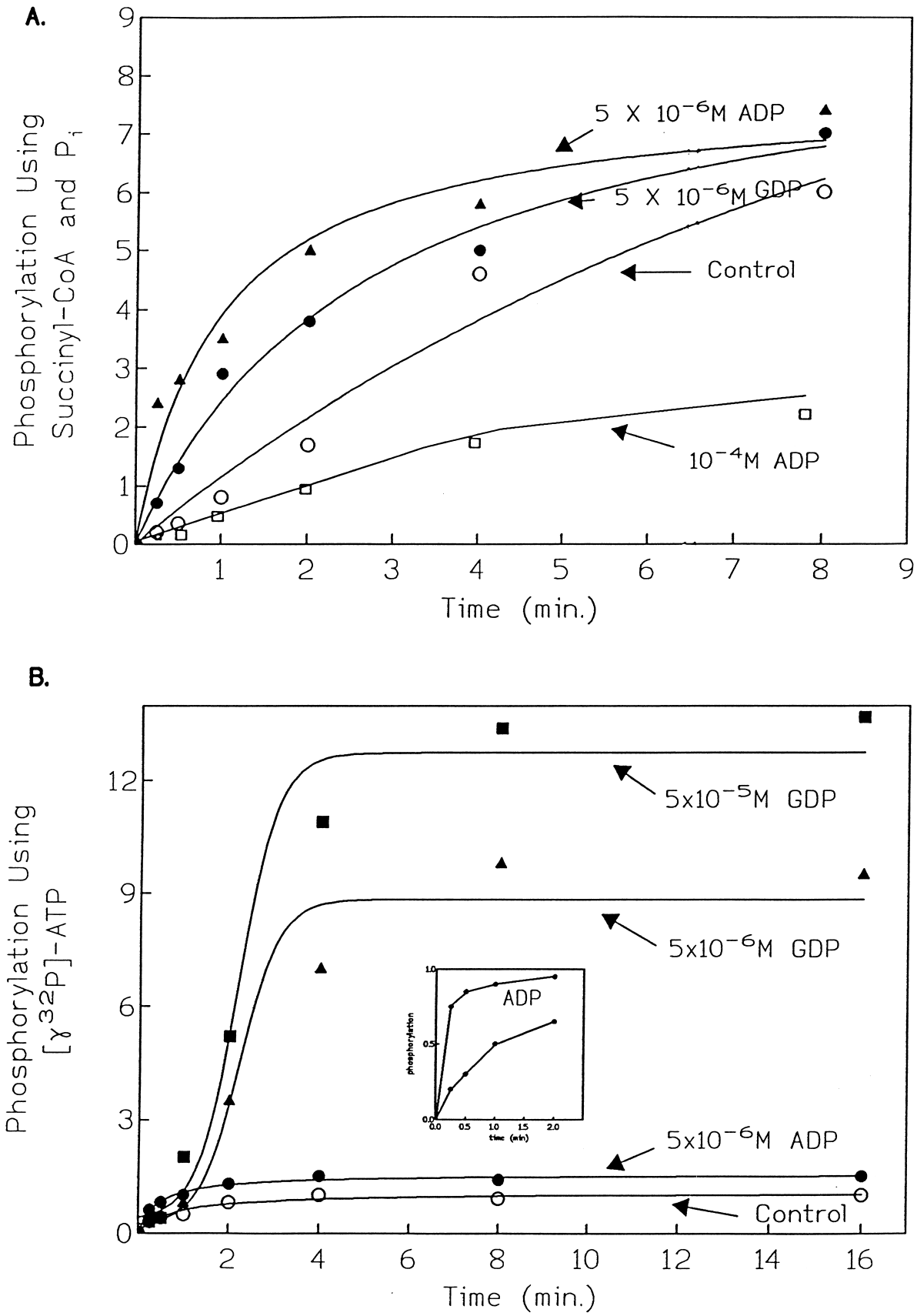

FIG. 1. Effects of NDP on the phosphorylation of *E. coli* SCS. *E. coli* lysates were incubated with 10^{-8} M succinyl-CoA and 10^{-8} M $^{32}P_i$ (A) or with 10^{-8} M [γ^{32}P]ATP (B). Reactions were terminated at the indicated times and analyzed by SDS-PAGE and autoradiography. Relative amounts of radiolabel incorporated into the α subunit of SCS were determined by densitometry and expressed in arbitrary units. When NDP was present, it was added at the indicated concentrations. Protein concentration was 1 mg/ml. The data are representative of three experiments.

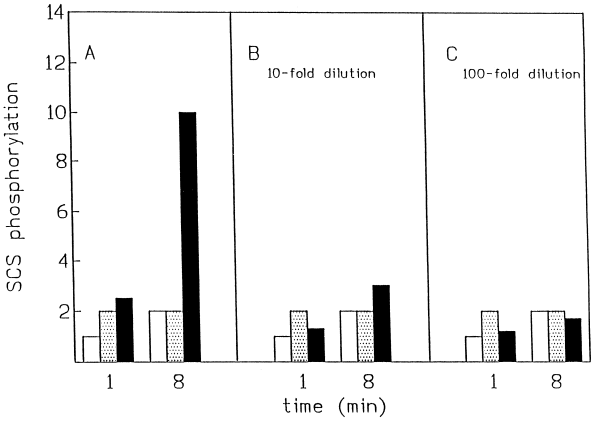

FIG. 2. Effect of sample dilution on the ability of GDP to enhance the fraction of *E. coli* SCS phosphorylated. *E. coli* lysates were incubated with 10^{-8} M [γ-^{32}P]ATP in the absence of any additions (open bars), with 5×10^{-5} M ADP (speckled bars), or with 5×10^{-5} M GDP (solid bars). Incubations were performed for 1 and 8 min. The latter period assured that maximum phosphorylation was achieved under all conditions. Protein concentration was 1 mg/ml (A), 0.1 mg/ml (B), or 0.01 mg/ml (C). Reactions were analyzed as for Fig. 1. Phosphorylation is expressed in arbitrary units. For each of the dilutions, the level of phosphorylation seen in the control sample at the 1-min incubation time was assigned a value of 1. The data are representative of two experiments.

formation, using enzyme preparations partially purified by hydroxyapatite chromatography and similarly diluted (data not shown).

The component necessary for the GDP effect functions without altering the level of phosphate donor. Although the *E. coli* enzyme preferentially utilizes adenine nucleotides, it can also use, to a lesser extent, guanine nucleotides (10, 13). In our *E. coli* extracts, this was verified by demonstrating that SCS could be phosphorylated with [γ-^{32}P]GTP (Fig. 3) when no [γ-^{32}P]ATP was produced, as determined by thin-layer chromatography. Characteristic of an autocatalytic reaction, the apparent rate of phosphorylation by GTP was not altered by sample dilution. Given the ability of GTP to serve as a phosphate donor, we could explain the GDP stimulation of the amount of phosphoenzyme formed if GDP were converted to radiolabeled GTP in a manner that would generate a net gain in substrate for phosphorylation. This could occur, for example, if GDP served as a phosphoacceptor from another phosphorylated protein(s). However, the stimulatory effects of GDP on SCS phosphorylation were observed in the absence of any other detectable protein phosphorylation (see Fig. 4). Alternatively, GTP may be formed from GDP and any $^{32}P_i$ produced as a result of ATP hydrolysis. However, as mentioned earlier, incubation of the cell lysates with $^{32}P_i$ and GDP did not lead to the phosphorylation of the enzyme, indicating that if any [γ-^{32}P]GTP was formed from these components, it was not sufficient to support the phosphorylation of SCS. We also considered the possibility that GDP inhibits an ATPase, thereby stabilizing the substrate for autophosphorylation. The half-life of the ATP in the reaction was monitored as described in Materials and Methods, and we did not detect an altered stability of [γ-^{32}P]ATP when reactions were performed in the presence of GDP. This aspect was further evaluated by examining of GDP could increase the fraction of SCS phosphorylated when GTP was used as a substrate. We reasoned that if GDP could inhibit an ATPase, it would be unlikely to also inhibit a GTPase. Again, high levels of GDP increased the amount of phosphoenzyme formed when GTP was used as a substrate, and its presence did not alter the stability of that substrate (Fig. 3). It has been reported that some NDP kinase is tightly associated with the SCS (9), making it possible that the NDPK preferentially binds the ATP, limiting its use for SCS autophosphorylation. Under such circumstances, however, we would not expect this inhibition to be relieved by the addition of GDP. That would stimulate the conversion of the ATP to GTP, providing a less favored substrate for SCS phosphorylation. In general, the sequestration of ATP by an ATP binding protein and its release by GDP is an unlikely scenario, since ADP could not elicit the same response as GDP. On the basis of these findings, it seems unlikely that the protein concentration dependence of the GDP effect on the amount of SCS phosphorylated reflects reactions that either alter the level of the phosphate donor or provide alternative substrates.

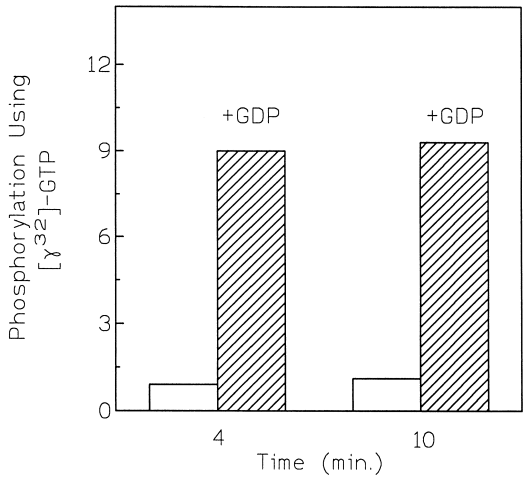

FIG. 3. SCS phosphorylation by GTP. *E. coli* lysates were incubated with 10^{-8} M GTP in the absence of any additions (open bars) or with 5×10^{-5}M GDP (striped bars). Incubations were performed for 4- and 10-min periods to assure that maximum levels of phosphorylation had been achieved. Protein concentrations was 1 mg/ml. Reactions were analyzed as for Fig. 1. The data are representative of two experiments.

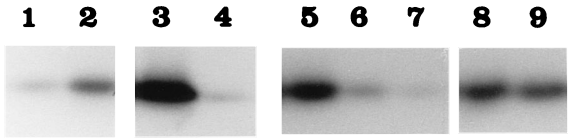

FIG. 4. Ammonium sulfate fractionation and reconstitution of the effector. Cell lysate (0.5 ml) was fractionated with 50% ammonium sulfate as described in Materials and Methods. The volumes of the resulting pellet and supernatant were adjusted to 0.5 ml to maintain a constant SCS concentration, assuming 100% recovery in the fraction. Unfractionated lysate (lanes 1 and 2) and the 50% ammonium sulfate supernatant (lanes 3 and 4) were phosphorylated with [γ-^{32}P]ATP in the absence (lanes 1 and 3) or presence (lanes 2 and 4) of 2×10^{-5} M GDP. For reconstitution experiments, 2 μl of the supernatant fraction was incubated with 16 μl of the pellet fraction to achieve an eightfold excess of pellet material to SCS compared with the ratio in the unfractionated samples. Samples were incubated for 0 min (lane 5), 60 min (lanes 6 and 8), or 90 min (lanes 7 and 9) and then phosphorylated in the absence (lanes 6 and 7) or presence (lanes 8 and 9) of 2×10^{-5} M GDP. The data are representative of three experiments.

Ammonium sulfate precipitates the GDP-regulated effector. We have determined that the effector responsible for the GDP-mediated increase in the level of SCS phosphorylation by ATP can be precipitated by 50% ammonium sulfate. At this concentration of ammonium sulfate, SCS remains soluble. Little or no phosphorylation of SCS was seen in the pellet fraction. Phosphorylation of SCS in the supernatant was signifi-

cantly higher than that seen in equivalent unfractionated samples (Fig. 4, lanes 1 and 3). In addition, the fraction of SCS phosphorylated was no longer enhanced by the addition of high concentrations of GDP. Instead, SCS phosphorylation was now significantly inhibited (Fig. 4, lanes 2 and 4). This effect of GDP is the more expected result when GDP concentrations are sufficient to allow its binding to the catalytic site (5, 14). Reconstitution experiments indicated that the 50% ammonium sulfate pellet contained a component that decreased the level of SCS phosphorylation in a time-, temperature-, and concentration-dependent manner. As an example, Fig. 4 (lanes 5 to 7) shows the amount of SCS phosphorylated when the 50% ammonium sulfate supernatant fraction was reconstituted with an eightfold excess of the pellet fraction for increasing times prior to phosphorylation with ATP. By 60 min of preincubation, the amount of SCS phosphorylated by ATP was significantly reduced, and it was negligible by 90 min. Figure 4 (lanes 8 and 9) also demonstrates that upon reconstitution with the pellet fraction, the amount of SCS phosphorylated could again be enhanced by the addition of GDP. The extent to which GDP could enhance the amount of SCS phosphorylated was the same in samples that had been preincubated for 60 or 90 min with the ammonium sulfate pellet fraction. These data indicate that the GDP-mediated increase in the fraction of SCS phosphorylated requires a component present in the ammonium sulfate pellet fraction and that the inhibition observed upon reconstitution with that fraction does not reflect the proteolytic degradation of SCS. It should be noted that although the level of SCS phosphorylation achieved in the 50% ammonium sulfate supernatant fraction seemed quite stable even after prolonged periods of storage, the inhibitory activity in the pellet fraction was sensitive to freeze-thaw or to storage, particularly if protease inhibitors were absent from the storage buffer.

The effector also inhibits SCS dephosphorylation by ADP. In the presence of the inhibitor, ATP is a poor substrate for SCS autophosphorylation, suggesting that it may function to inhibit ATP binding to the catalytic site. If so, we would expect that ADP binding to the catalytic site would also be inhibited. This was assessed by examining if high concentrations of ADP could elicit the dephosphorylation of the phosphoenzyme in the presence of the inhibitor. For this experiment, the enzyme present in the ammonium sulfate supernatant fraction was phosphorylated and then reconstituted with increasing amounts of the ammonium sulfate pellet fraction containing the effector. Afterwards, 5×10^{-4} M ADP was added to stimulate enzyme dephosphorylation. As seen in Fig. 5B, SCS that had not been reconstituted with the inhibitory fraction was rapidly dephosphorylated upon the addition of ADP (lane 5). A similar result was obtained with samples that had been reconstituted with a low level of the inhibitory fraction (Fig. 5B, lane 6). Samples that had been reconstituted to achieve the same ratio of inhibitor to SCS present in unfractionated lysates (Fig. 5B, lane 7) or with a twofold excess of inhibitor (lane 8) responded poorly to the addition of ADP, such that the majority of the protein remained phosphorylated during the time course of the experiment. In no case was dephosphorylation observed in the absence of added ADP (Fig. 5B, lanes 3 and 4). Figure 5A shows the extent of reconstitution achieved upon the addition of increasing amounts (up to a twofold excess) of the inhibitory fraction. Reconstitution is reflected by the decrease in the level of SCS phosphorylation by ATP. The data indicate that the effector present in the ammonium sulfate pellet limits the ability of ADP to function at the catalytic site to elicit the dephosphorylation of SCS.

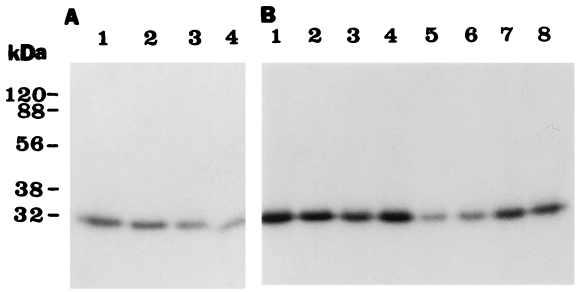

FIG. 5. The effector limits ADP-mediated SCS dephosphorylation. (A) The 50% ammonium sulfate supernatant fraction was incubated for 3 h in the absence (lane 1) or presence (lanes 2 to 4) of a 0.5-, 1-, or 2-fold excess of pellet material relative to SCS compared with the ratio in unfractionated samples. Samples were then phosphorylated with [γ-^{32}P]ATP to evaluate the extent of reconstitution of SCS with the inhibitor. (B) The 50% ammonium sulfate supernatant was phosphorylated with [γ-^{32}P]ATP and then incubated for 3 h with a 0.5-fold (lane 6), 1.0-fold (lane 7), or 2.0-fold (lanes 2, 4, and 8) excess of pellet fraction. Controls (lanes 1, 3, and 5) were incubated in the absence of pellet material. Lanes 1 and 2 show the level of phosphoenzyme present after the initial 3-h incubation without and with pellet fraction, respectively. After that time, 5×10^{-4} M ADP was added for 1 min (lanes 5 to 8). Lanes 3 and 4 represent the same samples as in lanes 1 and 2, but they were incubated for an additional 1-min period without added ADP. The samples were analyzed by SDS-PAGE and autoradiography to determine radiolabeling of the α subunit of SCS. The data are representative of two experiments.

DISCUSSION

The reaction catalyzed by SCS has generally been regarded to be driven by the availability of its substrates, and thus the enzyme has not been considered an important control point in the citric acid cycle. Such a passive role for the enzyme seems inconsistent with the complexity of its catalytic mechanism and the fact that it is the only enzyme in the citric acid cycle directly responsible for the production of NTP. The data presented in this work describe several previously unappreciated mechanisms by which *E. coli* SCS is regulated with regard to both the apparent level and rate at which catalysis can occur.

Product inhibition studies have previously shown that NDP and NTP share a common binding site on the enzyme (6). ADP binding to the catalytic site of SCS inhibits its autophosphorylation by ATP and stimulates the dephosphorylation of preformed phosphoenzyme (5, 14). These results were obtained in our experiments when ADP concentrations were above 5×10^{-6} M. However, lower concentrations (with as much as 100-fold) of ADP increased the rate of autophosphorylation of the α subunit of the *E. coli* enzyme about seven- to eightfold without significantly altering the fraction of protein phosphorylated. The stimulation of the rate of SCS phosphorylation was demonstrated under conditions that are not explained by substrate synergism, e.g., at subsaturating levels of substrates and not involving synergistic substrates. ADP was able to affect the rate of the reaction when either ATP or succinyl-CoA plus P_i were used as substrates. This argues that ADP does not stimulate the rate of phosphoenzyme formation by altering-interacting with a particular substrate of the reaction. Since no other phosphoprotein was detected under our reaction conditions when either ATP or succinyl CoA and P_i were used as substrates, ADP does not stimulate the phosphotransfer to SCS from a common intermediate. That an additional component participates in this phenomenon is also counterindicated by the observations that the effect of ADP was not diminished by sample dilution and/or by partial purification of the enzyme. In light of these data, it seems reasonable to propose that at low concentrations, ADP functions to enhance the rate of A-form SCS phosphorylation by binding to a high-affinity reg-

ulatory site on the enzyme, as previously demonstrated for G-form enzymes with GDP. The rate of autophosphorylation of the *E. coli* enzyme was also stimulated by low concentrations of GDP, but it was not as effective as ADP, resulting in a two- to threefold stimulation compared with the four- to eightfold stimulation seen with ADP. Thus, the nucleotide specificity of the regulatory binding site parallels that of the catalytic site.

In *E. coli* cells, there appears to be an additional level at which the activity of SCS is regulated. High levels of GDP, which were expected to mimic the effects of high ADP concentrations, bind to the catalytic site, and limit phosphoenzyme formation, actually enhanced the fraction of protein phosphorylated in cell lysates. This contrasts with the effect mediated by the ADP regulatory site which altered the rate, but not the amount, of SCS phosphorylated. Also in contrast to the latter, the ability of GDP to increase the amount of SCS phosphorylated was dependent upon protein-protein interactions, i.e., it was readily eliminated upon sample dilution, was sensitive to proteolytic degradation, and was lost upon partial purification of SCS by ammonium sulfate fractionation. Ammonium sulfate fractionation effectively removed a negative effector of SCS phosphorylation, resulting in elevated levels of phosphoenzyme compared with those for unfractionated samples.

A significant feature of the GDP stimulation of the amount of SCS phosphorylated is that it appears to regulate only one of the partial reactions. High concentrations of GDP stimulated the fraction of phosphoenzyme formed only when ATP (or GTP) was used as a phosphate donor but not when succinyl-CoA and P_i were employed. Given this observation, we considered the possibility that GDP alters the level and/or stability of the substrate, but we were unable to obtain evidence for that possibility. The sum of the data indicates that the ability of high GDP concentrations to enhance the fraction of phosphorylated SCS reflects its ability to remove or otherwise inactivate a negative effector of the enzyme's activity. In the absence of added GDP, the level of phosphoenzyme achieved within the first few minutes of the phosphorylation reaction would reflect the population of SCS free of inhibitor. The apparent plateau seen after that time is readily explained by the limited dissociation of the inhibitor from the enzyme under the experimental conditions used. Concentrations of GDP less than 5×10^{-6} M stimulate the rate of phosphoenzyme formation without significantly altering the inhibitor-enzyme association, as indicated by the minor change in the apparent level of phosphoenzyme under those conditions. Higher GDP concentrations would shift the equilibrium of inhibitor and enzyme toward free enzyme, as evidenced by the 7- to 10-fold increase in the level of phosphoenzyme. Those high concentrations of GDP, however, also compete with ATP at the catalytic site to limit phosphoenzyme formation. Thus, the apparent level achieved under such conditions is determined by two opposing reactions. This statement is consistent with the finding that when the inhibitor was eliminated by an alternative means, i.e., ammonium sulfate fractionation, even higher levels of SCS phosphorylation were seen compared with those achieved when high concentrations of GDP were added. Additionally, phosphoenzyme formation in the ammonium sulfate fraction exhibited the expected inhibition by high concentrations of GDP.

The finding that high GDP concentrations were effective in inhibiting SCS phosphorylation only when the effector was eliminated would suggest that the effector limits nucleotide binding to the catalytic site. This premise is supported by the observation that, in the presence of the inhibitor, ADP at high concentrations showed a limited ability to function at the catalytic site to elicit a dephosphorylation of the phosphoenzyme. Since the catalytic binding site for NTP-NDP is distinct from the binding sites for succinyl-CoA and P_i (5), the effector need not inhibit the phosphorylation achieved by those latter substrates.

In the citric acid cycle, SCS converts succinyl-CoA to succinate and in so doing carries out the only substrate-level phosphorylation that occurs in the cycle, resulting in the production of a high-energy nucleotide. Its reaction is totally reversible, with a ΔG° of -0.7 kcal mol^{-1}, so it may also readily function to replace the supply of succinyl-CoA, e.g., for anabolic purposes. The reversibility of the reaction renders it sensitive to the concentrations of its substrates. Our finding that an ADP regulatory site influences the rate of SCS phosphorylation places it in the category of other key metabolic enzymes that are feedback regulated. Such control allows for the rate of the reaction to be regulated constantly and automatically in response to immediate changes in cellular metabolism. We have also demonstrated that the enzyme is regulated at an additional level. An effector, readily separated from SCS, specifically limits the enzyme's use of nucleotides and therefore the ability of SCS to undertake the initial partial reaction, NTP + E $\leftrightarrows$ NDP + E~P. Restricted in its use of ATP as a substrate or of ADP for enzyme dephosphorylation, the catalytic capacity of SCS would be limited to the interconversion of succinyl-CoA and succinate when the effector is present. This provides SCS with the potential to regulate those metabolites independently of ATP, placing it in a key position for uncoupling carbohydrate and energy metabolism. The ability to uncouple the ATP-ADP and succinate–succinyl-CoA metabolisms may be the basis for the relatively complex mechanism by which SCS catalyzes its overall reactions. The potential for such regulation, however, would only be realized when cellular GDP concentrations are low, since the effector is inactive at high GDP concentrations. The presence of these regulatory mechanisms underscores the importance of SCS in the citric acid cycle and for determining the cell's potential to meet its metabolic demands.

How GDP functions to relieve the effector-mediated inhibition of SCS phosphorylation by ATP is, as yet, unclear. It may be that the effector is a guanine nucleotide binding protein whose association with SCS is altered upon GDP binding. It is also not clear to what extent this regulation is specific to A-form enzymes. In previous experiments examining the regulation of SCS phosphorylation in *D. discoideum*, we did not detect an increase in the level of protein phosphorylation upon the addition of high levels of either GDP or ADP (1, 18). However, such experiments examined the enzyme released from mitochondria that were disrupted in the absence of detergent. Those conditions may not have been suited to the coincident release of the effector. Alternatively, the relative concentration of the effector in our preparations may not have been sufficient to observe its effects. This is particularly pertinent, since the data obtained with the *E. coli* enzyme suggest that the inhibitory effects are sensitive to sample dilution. Having established a reconstitution system to monitor the presence of the effector, we are now focusing our attention toward its purification and identification. In so doing, we expect to discern how it mediates its effects on SCS and if homologs exist in other systems.

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