Evidence for allosteric regulation of succinyl-CoA synthetase

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We have previously reported that distinctly different concentrations of GDP stimulate the phosphorylation and dephosphorylation of p36, the α -subunit of succinyl-CoA synthetase (SCS) in *Dictyostelium discoideum*. In this present study, we have investigated the mechanism underlying these dual effects of GDP. Dephosphorylation of p36 is induced by relatively high levels of GDP and is coincident with the formation of GTP. This indicates that, at high concentrations, GDP serves as a substrate of SCS. However, 100-fold lower concentrations of GDP, which do not bind to the catalytic site to induce SCS dephosphorylation, stimulate p36 phosphorylation. This stimulation is not

diminished by dilution of the sample, and is retained during purification of the protein. Gel-filtration analyses indicate that SCS in our system behaves as a non-interacting $\alpha\beta$ dimer, the hydrodynamic behaviour of which is not altered by the presence of added GDP. The data indicate that altered protein–protein interactions do not account for the stimulation of p36 phosphorylation by low GDP concentrations. We propose that GDP functions as an allosteric regulator of SCS, and experiments using guanosine 5'-[β -thio]diphosphate (GDP[S]) are shown to distinguish further the allosteric and catalytic binding sites.

INTRODUCTION

Previous experiments from this laboratory (Anschutz et al., 1989; Um and Klein, 1991) have described the rapid phosphorylation and dephosphorylation of a 36 kDa protein (p36) in extracts of *Dictyostelium*. Phosphorylation of p36 was achieved preferentially using GTP, as opposed to ATP, as the phosphate donor, and was stimulated by the specific addition of relatively low concentrations of GDP. GDP primarily enhanced the rate of p36 phosphorylation and seemed to do so by decreasing the apparent K_m of p36 for nucleotide triphosphate (Anschutz and Klein, 1990). The ability of low concentrations of GDP to stimulate the rate of p36 phosphorylation was developmentally regulated: crude sample preparations from aggregation-competent cells were sensitive to GDP, whereas crude preparations from vegetable cells were not. When dephosphorylation of p36 was studied, GDP was also found to stimulate p36 dephosphorylation and did so in all cell preparations tested (Um and Klein, 1991). The concentrations of GDP required for dephosphorylation were relatively high and distinct from those required for enhanced phosphorylation of p36. We had proposed, on the basis of these observations, the existence of two GDP-binding sites that regulated p36 phosphorylation: one of high affinity the occupancy of which stimulates the phosphorylation, the other of low affinity which is involved in dephosphorylation.

Recently, we have purified and identified p36 as the α subunit of succinyl-CoA synthetase (SCS) (Anschutz et al., 1993). This enzyme has been isolated from both prokaryotic (Gibson et al., 1967; Ramaley et al., 1967) and eukaryotic sources (Cha and Parks, 1964a; Ball and Nishimura, 1980), and catalyses the reactions:

As seen in this scheme, the phosphoenzyme is formed as an

intermediate of the overall reaction. SCS from different sources displays differences in nucleotide specificity and quaternary structure, although any correlation between these two properties is unclear. In *Escherichia coli*, the enzyme exists in an $\alpha_2\beta_2$ tetramer (Krebs and Bridger, 1974; Wolodko et al., 1986), and preferentially utilizes adenine nucleotides as substrates (Murakami et al., 1972; Kelly and Cha, 1977). In contrast, the enzyme from pig heart is an $\alpha\beta$ dimer (Cha et al., 1967; Wolodko et al., 1986) and is guanine nucleotide-specific (Sandai et al., 1956). Regardless of these differences, SCS from these two different sources displays similar and unusual catalytic properties, termed substrate synergism (Bridger et al., 1968). This describes the fact that the presence of a substrate for one partial reaction stimulates another partial reaction. In the case of *E. coli* SCD, which exists as a tetramer with two catalytic sites, the phenomenon was explained by alternating sites having catalytic co-operativity (Bild et al., 1980). In this model, binding of a substrate at one catalytic site facilitates catalytic events at the other catalytic site. However, this model is not applicable to the pig heart SCS which is a dimer with one catalytic site. In this case, a same-site model has been proposed (Nishimura and Mitchell, 1985), whereby binding of a substrate to the catalytic site enhances another partial reaction occurring at that site. The stimulation of SCS phosphorylation by GDP has not been reported in any other system and is a phenomenon that is not explained by the current models for SCS regulation. The work presented in this paper was performed to understand the mechanism by which GDP enhances the rates of both phosphorylation and dephosphorylation of SCS, in an attempt to define and compare the binding sites involved in each reaction. The results support the existence of an allosteric regulatory site for GDP on SCS.

MATERIALS AND METHODS

SCS preparation

Ax-2 amoebae (Watts and Ashworth, 1970) were grown in HL-5 medium (Sussman, 1966). Preparation of crude membrane

Abbreviations used: SCS, succinyl-CoA synthetase; GDP[S], guanosine 5'-[β -thio]diphosphate; ATP[S], adenosine 5'-[γ -thio]triphosphate; GTP[S], guanosine 5'-[γ -thio]triphosphate.

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fractions enriched in p36 is described elsewhere (Um and Klein, 1991). To prepare mitochondria, cells were lysed in buffer A (70 mM sucrose, 220 mM D-mannitol, 2 mM Hepes, pH 7.4, 1 mM EDTA, and 0.5 mg/ml BSA). After removal of cell debris and nuclei by centrifugation at 484 *g* (2000 rev./min) for 10 min, the lysates were centrifuged for 20 min at 9800 *g* (9000 rev./min) in a Beckman JA-20 rotor. The pellet was gently resuspended in buffer A and re-centrifuged. The pellet of this second centrifugation was enriched in mitochondria, as determined by the recovery of succinate dehydrogenase activity and the lack of alkaline phosphatase, phosphodiesterase and lactate dehydrogenase activities. To release p36 into a soluble form, samples were frozen and then thawed in an equal volume of 10 mM Tris/HCl, pH 8.0, containing 1 mM EDTA, 1 mM EGTA, and 0.4 mM dithiothreitol. Samples were then centrifuged at 100 000 *g* for 1 h at 4 °C. Under these conditions, approx. 60 % of the p36 in the original membrane or mitochondrial preparations was released into the supernatant fraction. Unless indicated otherwise, this fraction was dialysed against buffer [50 mM Tris/HCl, pH 8.0, 10 mM MgCl₂, and 10 % (v/v) glycerol] and stored at -20 °C until further use. More purified preparations of p36, referred to in the text as partially purified, were obtained by chromatography on hydroxyapatite and Affi-Gel Blue (Bio-Rad). Such samples contained seven to nine silver-stained bands on SDS/PAGE (Anschutz et al., 1993). In addition to the α - and β -subunits of SCS, three to four other bands could be considered as the major components in the preparation.

Phosphorylation and dephosphorylation assays

All of the assays were performed at room temperature in 50 mM Tris/HCl, pH 8.0, containing 10 mM MgCl₂. Unless otherwise indicated, endogenous substrates were removed from all SCS samples by dialysis or during additional purification steps. Phosphorylation was initiated by incubating samples with 10 nM [γ -³²P]GTP (1.0 Ci/ μ mol). Samples prepared in this manner could be monitored for p36 phosphorylation in the absence of any endogenous dephosphorylation, previously demonstrated in cruder preparations (Anschutz et al., 1989). To monitor dephosphorylation, the phosphorylation reaction was stopped by the addition of 30 mM EDTA and dialysed against buffer [50 mM Tris/HCl, 1 mM EDTA and 10 % (v/v) glycerol] to remove free radionucleotides. Dephosphorylation was initiated by adding 10 mM MgCl₂ and 0.1 mM GDP. Radiolabel in p36 was determined by SDS/PAGE and autoradiography of gels that had been fixed for 30 min in 15 % (v/v) methanol (Laemmli, 1970). The presence of acetic acid was avoided as this treatment resulted in the removal of all the radiolabel in p36. Histidine phosphate is acid labile (Fujitaki and Smith, 1984), and SCS is known to be phosphorylated on a histidine residue in the α subunit (Kreil and Boyer, 1964). Relative intensities of p36 radiolabelling in linear-range autoradiograms were determined using the Bio-Rad model 620 video densitometer and 1-D Analyst software. Initial apparent rates of p36 phosphorylation were determined by analysing the reaction after 5, 10, 15 and 30 s of incubation to ensure linearity of the reaction. Protein determinations were made by the method of Lowry et al. (1951).

T.l.c.

The dephosphorylation reactions were terminated by the addition of 30 mM EDTA and quick-frozen in solid CO₂/ethanol. Equal volumes of each reaction mixture were chromatographed on

0.1 mm cellulose MN 300 poly(ethyleneimine) plates in 0.75 M KH₂PO₄, pH 3.4. Spots corresponding to radiolabelled GTP, ATP and P_i were identified by autoradiography.

Gel-filtration chromatography

A Sephacryl S-200 column (1 cm $\times$ 40 cm) was equilibrated at room temperature in buffer B [50 mM Tris/HCl, pH 8.0, 10 mM MgCl₂, 10 % (v/v) glycerol, 0.02 % sodium azide and protease inhibitors (Meier and Klein, 1988)]. The void volume was determined with Blue Dextran 2000 and the column was calibrated with the following protein standards of known Stokes radius (Pharmacia); RNAase A, 1.6 nm; chymotrypsinogen A, 2.1 nm; ovalbumin, 3.1 nm; BSA, 3.6 nm; aldolase, 4.8 nm; catalase, 5.2 nm; ferritin, 6.1 nm and thyroglobulin, 8.5 nm. The soluble fraction of p36 was prepared in buffer B and applied in a volume of 0.15 ml for small-zone gel filtration or 6.5 ml for large-zone filtration. Fractions (0.25 ml) were collected at a flow rate of 10–15 ml/h. Elution profiles of p36 were determined by phosphorylating every other fraction with [γ -³²P]GTP or by immunoblotting. Alternatively, the soluble fraction that had been phosphorylated with [γ -³²P]GTP was chromatographed. In this case, MgCl₂ in the buffers was replaced by 1 mM EDTA to prevent dephosphorylation of p36. Every other fraction was analysed by SDS/PAGE and autoradiographed to obtain the elution profile of p36.

Immunoblotting

Gel-filtration fractions were analysed by SDS/PAGE and blotted on to Immobilon (Millipore) in the presence of 0.01 % SDS by the method of Towbin et al. (1979). Membranes were incubated at 37 °C in blocking solution composed of 5 % (v/v) non-fat dry milk/10 % (v/v) IgG-free horse serum in Tris-buffered saline (20 mM Tris/HCl, pH 8, 150 mM NaCl), for 30 min, and then incubated overnight with antiserum raised against a synthetic peptide of the N-terminal 18 amino acids of p36 (A. Anschutz and C. Klein, unpublished work). After washing four times (15 min each) with Tris-buffered saline, the filter was incubated with ¹²⁵I-protein A (10⁶ c.p.m./ml) in blocking solution. The filter was then rinsed again with Tris-buffered saline (four times 15 min each) and autoradiographed. Relative intensities of the bands corresponding to p36 were analysed by densitometry.

Materials

[γ -³²P]GTP was purchased from ICN. Sephacryl S-200 and Blue-Dextran 2000 were obtained from Pharmacia. Centricon 10 microconcentrators were obtained from Amicon. All other chemicals were purchased from Sigma.

RESULTS AND DISCUSSION

Using crude membranes prepared from vegetative or starved cells, we have previously shown that relatively high concentrations of GDP inhibit the phosphorylation of p36 by GTP and stimulate the dephosphorylation of the phosphoprotein (Um and Klein, 1991). The same concentrations of ADP were ineffective. Given the identification of p36 as the α subunit of SCS (Anschutz et al., 1993), it is likely that these effects reflect the binding of GDP to the catalytic site [see eqn. (1)]. Consistent with this interpretation of the effects of high concentrations of GDP is the finding that GDP still stimulates p36 dephosphorylation in partially purified preparations (Figure 1a), suggesting that GDP acts directly on SCS to elicit its dephosphorylation. Almost all of

Figure 1 Formation of GTP during dephosphorylation of p36 by GDP

Partially purified preparations of p36 were phosphorylated with [γ - 32 P]GTP. Dephosphorylation was monitored in samples incubated in the absence (lanes 1–3) or presence (lane 4) of 0.1 mM GDP. Samples were taken before (lane 1) and 15 s (lanes 2 and 4) and 2 min (lane 3) after dephosphorylation was initiated. At such times, samples were mixed with excess EDTA to terminate the reaction and analysed by SDS/PAGE (a) or t.l.c. (b) and autoradiography.

Figure 2 Dialysis of vegetative cell preparations rendering the phosphorylation of p36 sensitive to GDP

Mitochondria-released preparations from vegetative cells were phosphorylated with [γ - 32 P]GTP before (a) or after (b) dialysis. The phosphorylation was performed in the absence (odd-numbered lanes) or presence (even-numbered lanes) of 2 μ M GDP. Reactions were terminated at 10 s (lanes 1 and 2), 30 s (lane 3 and 4) or 60 s (lanes 5 and 6). Samples were analysed by SDS/PAGE and autoradiography.

the radiolabel in p36 was removed within 15 s of the addition of 0.1 mM GDP (lane 4). In the absence of added GDP, no dephosphorylation of the protein was detected, even after several minutes of incubation (lane 3). In keeping with the proposed reaction mechanism for this enzyme, we also showed that dephosphorylation of the enzyme in response to high concentrations of GDP resulted in the production of GTP (Figure 1b). T.l.c. analyses indicated that GTP was formed rapidly when phosphorylated p36 was incubated with GDP (lane 4). As SCS is the predominant, if not only, protein that is phosphorylated under our conditions, it is unlikely that GTP is produced by the dephosphorylation of another protein. In that regard, it should be noted that no GTP was formed in samples incubated in the absence of added GDP (lanes 2 and 3), conditions under which no dephosphorylation of p36 occurred. From these results we conclude that high concentrations of GDP act as a substrate of SCS and, by binding to the catalytic site, elicits the dephosphorylation of the enzyme.

In contrast with the effects of high concentrations of GDP, low

concentrations ($\leq 2 \mu$ M) do not stimulate any dephosphorylation of p36, but actually enhance its rate of phosphorylation. An unusual feature of this stimulation is that it was found to occur in crude sample preparations from starved cells but not in similar preparations from growing cells (Anschutz and Klein, 1990; Um and Klein, 1991). This could reflect a difference in the enzyme produced at these two stages, or it could indicate a developmental change in a regulatory component or molecule. Such a regulatory component could be GDP itself. To examine these possibilities, we prepared mitochondria from such cells, released their soluble contents by freeze/thawing in a hypotonic buffer, and then extensively dialysed the soluble fraction. As shown in Figure 2(a), the addition of 2 μ M GDP did not stimulate the phosphorylation of p36 in the undialysed sample. In this particular experiment, this concentration of GDP actually slightly reduced the apparent level of p36 phosphorylation. However, even lower concentrations of GDP still did not stimulate the phosphorylation of p36. In contrast, the dialysed sample showed an enhanced phosphorylation of p36 upon addition of low concentrations of GDP (Figure 2b). As in the case of starved cell preparations, GDP stimulated the initial rate of p36 phosphorylation (in this particular experiment, approx. 6-fold) and did not significantly alter the final level of phosphorylation. Therefore elimination of molecules smaller than 10 kDa renders the enzyme sensitive to the stimulatory effects of GDP.

The above-mentioned data indicate that the developmental changes in p36 phosphorylation are not due to inherent differences in the protein that is expressed in starved compared with growing cells. Thus other events were considered to explain the mechanism by which GDP stimulated p36 phosphorylation. We eliminated the possibility that 32 P_i generated by GTP hydrolysis was used to re-form [γ - 32 P]GTP via a GDP-dependent reaction. This was done by incubating extracts with 10 nM P_i and 2 μ M GDP. Under such conditions we did not observe any phosphorylation of p36 (results not shown). We then considered the possibility that GDP stimulated this process by dephosphorylating pre-existing enzyme and thus making available additional p36 for radiolabelling. This seemed unlikely for several reasons. Our samples are prepared at relatively high protein concentrations, conditions that we have shown previously to support the rapid dephosphorylation of p36 (Um and Klein, 1991). Thus we would expect little or no phosphoenzyme in these samples. Also, the low concentrations of GDP that stimulate p36 phosphorylation do not elicit its dephosphorylation, and no dephosphorylation is observed during the reaction. However, to ensure that the level of endogenous phosphoenzyme did not in some way influence the observations made, we performed the experiment shown in Figure 3. p36 preparations were preincubated in the presence of 0.1 mM GDP to ensure its total dephosphorylation. Afterwards, samples were extensively dialysed and then assayed for p36 phosphorylation in the absence or presence of 2 μ M GDP. The rate of p36 phosphorylation was still effectively stimulated by GDP in a manner that could not be distinguished from the preparation that had not been preincubated.

Nucleotides have been shown to alter the polymerization of specific proteins and it is clear that a change in protein–protein interactions could affect the ability of p36 to autophosphorylate. The possibility that GDP affected p36 phosphorylation by altering its association of subunits was examined by gel filtration. First we determined the subunit composition of the enzyme and any possible changes with respect to protein concentration using both small-zone and large-zone analysis. At a loading concentration of 0.05 mg/ml, the peak of p36 elution in a small-zone experiment coincided with that of BSA (Figure 4a). This

Figure 3 Preincubation does not alter the sensitivity of p36 phosphorylation to GDP

p36 preparations were preincubated in the absence (a) or presence (b) of 0.1 mM GDP. Samples were dialysed as described in the Materials and methods section and then phosphorylated with [γ - 32 P]GTP for 30 s (lanes 1 and 2), 1 min (lanes 3 and 4) and 2 min (lanes 5 and 6). 2 μ M GDP was present in the reactions (even-numbered lanes) or absent (odd-numbered lanes). Samples were analysed by SDS/PAGE and autoradiography.

corresponds to an apparent molecular size of 67 kDa (assuming a spherical shape of the protein). Our sucrose-density-gradient analysis (A. Anschutz and C. Klein, unpublished work) is consistent with this assumption. It would appear that the SCS in our system is composed of one molecule of p36 (the α -subunit) and one molecule of the β -subunit. This is the situation seen for guanine-nucleotide-specific forms of the enzyme in other eukaryotes (Ball and Nishimura, 1980; Wolodko et al., 1986). The migration of p36 did not change when the loading concentration was increased 100-fold to 5 mg/ml, suggesting that the dimer is the stable form of the enzyme. However, as samples are significantly diluted during their chromatography in such experiments, any tetramers that may have been present in the original sample could have dissociated during analysis. Therefore large-zone experiments were used to examine the subunit composition of p36 at 5 mg/ml. Figure 4(b) shows a typical elution profile. The boundaries of the profile were almost symmetrical, with no signs of heterogeneity. The mid-point of the advancing boundary was nearly identical with the point of peak elution of p36 in the small-zone experiments. These results indicate that, independent of protein concentration in our preparations, the dimeric form of SCS does not aggregate nor does it associate with another component of molecular size greater than 20 kDa. This latter qualification reflects the limits of detection of our assay.

In these experiments, the elution of p36 was monitored by either Western blots using a specific anti-p36 antibody or phosphorylating fractions with GTP and analysing them by SDS/PAGE and autoradiography. In the latter case, we also determined that GDP could still effectively stimulate the phosphorylation of p36 in fractions obtained in the small-zone experiments. This indicates that any component necessary to do so had not been separated from p36 during this procedure (which is also a purification step) and supports the hypothesis that this reflects a direct action of GDP on the protein. These findings also indicate that the protein was not denatured or otherwise altered during chromatography. A similar set of experiments was performed using samples of p36 that had been phosphorylated and then monitoring the elution of the radiolabelled protein. The results were identical with those obtained using the unlabelled

Figure 4 Gel-filtration chromatography of p36

(a) Small-zone experiments used 0.15 ml of p36 preparations at 0.05 mg/ml. (b) Large-zone experiments used 6.5 ml of sample containing 5.0 mg/ml. All samples had been extensively dialysed before fractionation on a 1 cm $\times$ 40 cm Sephacryl S-200 column. Fractions were analysed either by Western blot or phosphorylated with [γ - 32 P]GTP and analysed by SDS/PAGE and autoradiography to determine the elution profile of p36. Identical profiles were obtained when 2 μ M GDP was present in the chromatographed samples and buffers. The arrows indicate the peaks (a) or midpoints of the advancing boundaries (b) of three standards of known Stokes radius: aldolase (4.8 nm, fraction 19), BSA (3.6 nm, fraction 24) and ovalbumin (3.1 nm, fraction 31).

samples, indicating that the phosphorylation of p36 does not affect its subunit composition.

To assess if low concentrations of GDP can change the subunit composition of SCS or its association with another component, the same experiments were performed in the presence of 2 μ M GDP. For such experiments, this concentration of GDP was added to the sample which was chromatographed on columns equilibrated and developed with buffers containing the nucleotide. Under these conditions, no change in the elution profile of p36 was observed. (See Figure 4 for results representative of elutions carried out in the absence or presence of GDP.) This was true whether the sample had been phosphorylated before or after chromatography. We concluded that GDP does not stimulate the phosphorylation of p36 by changing the interaction of subunits of SCS or by causing the protein to associate with another cellular component.

These conclusions were further substantiated by the finding that partially purified preparations of p36 still responded to low concentrations of GDP by stimulating its rate of phosphorylation. This was true independent of the protein concentration of the sample. As shown in Figure 5, the addition of 2 μ M GDP enhanced p36 phosphorylation even when partially purified

Figure 5 Effect of sample dilution on the stimulatory effect of GDP

Partially purified preparations were phosphorylated with [γ - 32 P]GTP in the absence (odd-numbered lanes) or presence (even-numbered lanes) of 2 μ M GDP. The protein concentration in the reaction mixtures was 500 μ g/ml (a) and 5 μ g/ml (b). p36 phosphorylation was analysed after 15 s (lanes 1 and 2), 30 s (lanes 3 and 4), 1 min (lanes 5 and 6) and 2 min (lanes 7 and 8) of incubation. Times of exposure of autoradiograms were optimized for each experiment.

Table 1 Effect of GDP[S] on p36 phosphorylation and its stimulation by GDP

Initial rates of p36 phosphorylation were normalized by taking the rate measured in the absence of GDP and GDP[S] as 100. Values in parentheses are fold increases in the initial rate of p36 phosphorylation caused by the addition of GDP in the presence of the indicated concentration of GDP[S]. ND, not done.

GDP[S] (μ M)	Initial rates of p36 phosphorylation		
	GDP (M) ... 0	10^{-7}	5×10^{-7}
0	100	170 (1.7)	220 (2.2)
0.1	100	180 (1.8)	ND
0.2	72.5	ND	ND
2	57.0	ND	154 (2.7)
10	34.0	74.8 (2.2)	ND
5	6.7	14.7 (2.2)	15.4 (2.3)
100	4.4	ND	ND
200	0	ND	ND

samples were diluted 100-fold. In both cases, the rate of phosphorylation was stimulated 5–7-fold. The ability of GDP to stimulate the phosphorylation of p36 in more purified preparations and in a manner that is independent of protein concentration would argue that this is a direct effect of GDP on SCS itself and does not require another component or an aggregation of dimers. The latter conclusion is in agreement with our gel-filtration analysis, as well as our more recent observation that GDP stimulates the rate of phosphorylation by GTP of the purified pig heart SCS, which is also a guanine-nucleotide-specific enzyme (A. Anschutz and C. Klein, unpublished work).

The sum of the data presented in this paper would indicate that GDP functions at an allosteric site to stimulate p36 phosphorylation. The binding of GDP to such an allosteric site is clearly distinguished from its binding to the catalytic site. The latter was demonstrated to have a relatively low affinity for GDP and, when occupied, leads to the dephosphorylation of the enzyme and the formation of GTP. Previously we have reported that similar GDP concentrations inhibit the phosphorylation of p36 by GTP (Um and Klein, 1991). This result is expected, as GDP competes for GTP binding to the enzyme (Cha and Parks, 1964b), and also indicates that the low-affinity site for GDP binding represents the catalytic site. To provide additional support for our hypothesis that there also exists a separate high-

affinity site for GDP that allosterically regulates p36 phosphorylation, we examined the effects of GDP[S] on the phosphorylation of p36. In the case of the *E. coli* enzyme, which uses adenine nucleotides, it has been shown that ATP[S] can bind to the catalytic site and function to thiolate the α -subunit (Nishimura and Mitchell, 1984; Wolodko et al., 1983). We have previously shown that GTP[S] can effectively compete for GTP in the phosphorylation of p36, which is guanine-nucleotide-specific (Anschutz and Klein, 1990). As the catalytic site of SCS can recognize thiol analogues of nucleotides, we could expect that GDP[S] may also be able to bind to the catalytic site. This was examined by assessing the effects of GDP[S] on the phosphorylation of p36 by [γ - 32 P]GTP. As shown in Table 1, the addition of this compound inhibited the phosphorylation of p36. Inhibition was initially observed at 0.2 μ M GDP[S], and was complete at 0.2 mM. As GDP inhibition of p36 phosphorylation is initially observed at approx. 5 μ M, GDP[S] may actually bind somewhat better to the catalytic site. In contrast with its action at the catalytic site, GDP[S] did not affect the allosteric regulation of p36. Its addition, at any concentration, did not stimulate the rate of p36 phosphorylation. Nor was the ability of GDP to stimulate the rate of p36 significantly altered, even when GDP[S] was present at a 500-fold excess over GDP (Table 1). Under such conditions, GDP[S] concentrations were high enough for it to bind to the catalytic site and inhibit phosphorylation. These data indicate that GDP[S] does not efficiently compete with GDP at the regulatory site, another property that distinguishes this site from the catalytic site.

SCS is a critical enzyme in ketone-body metabolism, porphyrin biosynthesis and citric acid cycle metabolism. We have presented evidence that a high-affinity GDP-binding site allosterically regulates the activity of the enzyme, and thus the flux of metabolites through these pathways. How the binding of GDP to the allosteric site actually leads to an enhanced rate of SCS phosphorylation is not totally clear. Preliminary data have indicated that GDP probably alters the affinity of the enzyme for GTP (Anschutz and Klein, 1990). We are currently confirming this observation and investigating whether a change in substrate affinity is specific for the GTP-binding site, or whether a more global conformational change in the enzyme is induced that would influence its affinity for other substrates as well. Such lines of investigation will provide a better basis for understanding the mechanism by which GDP allosterically regulates the overall activity of this enzyme.

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