# Allosteric Regulation of the ATP Sulfurylase Associated GTPase†

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ABSTRACT: ATP sulfurylase catalyzes and chemically links the hydrolysis of GTP and the synthesis of activated sulfate (APS). Like many GTPases, its GTPase activity is allosterically regulated, in this case, by APS-forming reactants and their analogues. Using these activators, we have been able to mimic many of the complexes that form in the native reaction, including an E•AMP intermediate. The effects of each of these complexes on GTP hydrolysis are determined. The results of pre-steady-state and isotope trapping studies demonstrate that the binding of activator and substrate to the enzyme are near equilibrium and that the rate-determining step appears to be scission of the $\beta,\gamma$-bond of GTP. These properties of the system allow the energetic consequences of activator binding on the ground- and transition-state complexes to be evaluated. Activation occurs predominantly by transition-state stabilization, resulting in $k_{cat}$ increases. The values for $k_{cat}$ span a 180-fold range and vary with each activator. $K_m$, or ground-state, effects are relatively small, ~3-fold, and are uniform throughout the activator series. These studies provide an in-depth view of the energetic interactions between the two active sites at each step of the APS-forming reaction.

GTPase activation of target proteins is a well-established metabolic regulatory mechanism. ATP sulfurylase (ATP: sulfate adenylyltransferase, EC 2.7.7.4), isolated from *Escherichia coli K-12*, catalyzes two reactions: the activation of sulfate and the hydrolysis of GTP (Leyh & Suo, 1992) (eq 1 and 2). These reactions are kinetically and energetically coupled. At a saturating concentration of GTP, the initial rate of APS synthesis is stimulated 116-fold (Leyh & Suo, 1992), and the APS-forming reaction is shifted ($5 \times 10^6$)-fold toward APS synthesis (Liu et al., 1994). ATP sulfurylase appears to be a tetramer of heterodimers each composed of a smaller, CysD (35 kDa), and a larger, CysN (53 kDa), subunit. Sequence similarities with other GTPases suggest that CysN is the GTPase (Leyh et al., 1992); it is not yet clear whether CysD harbors the active site for APS synthesis. The kinetic mechanism (Liu et al., 1993) and the basis for the energetic linkage in this system (Liu et al., 1994) have been determined.

$$ATP + SO_4 \rightleftharpoons APS + PP_i \quad (1)$$

$$GTP + H_2O \rightleftharpoons GDP + P_i + H^+ \quad (2)$$

Sulfate is a stable, nonreactive compound that must be activated to participate in its subsequent metabolic reactions, reduction and transfer. It is generally accepted that the activation of sulfate is required for its assimilation in all organisms (De Meio, 1975; Siegel, 1975). Many bacteria, including *E. coli*, reduce sulfate to sulfide for the synthesis of cysteine and methionine (Siegel, 1975); others perform the evolutionarily ancient metabolic process of linking the reduction of sulfate to the electron transport chain (Peck, 1993). Peptidyl and carbohydrate sulfate esters are crucial for proper functioning of many aspects of mammalian metabolism and physiology: hemophilia (Leyete et al., 1991), growth factor recognition (Ishihara et al., 1993), hormonal regulation (Leyh, 1993), lymph cell circulation (Imai et al., 1993)—there are many examples (Lindahl & Hook, 1978; Mudd, 1980; Mulder & Meermen, 1982; Jakoby & Ziegler, 1990; Leyh, 1993). The sulfuryl donor used in these transesterifications is 3′-phosphoadenosine 5′-phosphosulfate, a phosphorylated form of APS (De Meio, 1975).

Typically, only the GTPase•GTP complex is competent to bind to and stimulate a target; activation ceases upon hydrolysis (Bourne et al., 1990, 1991). Thus, allosteric regulation of hydrolysis modulates the time interval during which a target may be activated (Hopfield, 1974; Bourne et al., 1990). Many GTPases exhibit a low, basal rate of GTP hydrolysis that is activated by other proteins, GAPs (GTPase-activating proteins) and/or GNRPs (guanine nucleotide release proteins) (Bourne et al., 1990). As their names imply, these proteins enhance turnover by increasing the rate of either the hydrolytic or the product release steps. ATP sulfurylase, too, has a low hydrolytic activity that can be activated; however, in this case, activation is induced by small molecules—the reactants of the APS-forming reaction. Many of the complexes that form during the native reaction cycle can be studied in isolation using individual reactants. The reaction coordinate for hydrolysis can be mapped onto that for APS synthesis by studying the effects of these complexes on GTP hydrolysis. Activator-induced alterations in $k_{cat}$ and/or $K_m$ for hydrolysis result from physical changes that stabilize, or destabilize, specific ground- and/or transition-state complexes in the catalytic cycle. Characterizing the effects of these activators on ground-state and kinetically significant transition-state complexes is the primary focus of this article.

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## MATERIALS AND METHODS

*Materials.* Nucleotides, NADH, and phosphoenolpyruvate (PEP) were the highest grades available from Sigma Chemical Co. Tricine[1]-stabilized radionucleotides [α-$^{32}$P]GTP (3000 Ci/mmol) and [γ-$^{32}$P]GTP (6000 Ci/mmol) were purchased from the DuPont NEN Corp. Pyruvate kinase (yeast) and lactate dehydrogenase (rabbit muscle) were purchased from Boehringer Mannheim Corp. The poly(ethylenimine)−cellulose-F thin-layer chromatography (PEI-F TLC) plates were obtained from E. Merck Co. Other compounds were reagent grade or better.

*ATP Sulfurylase.* The enzyme was purified according to a published protocol from an *E. coli K-12* strain containing an expression vector that results in the production of high levels of the *E. coli K-12* enzyme (Leyh et al., 1987). The specific activity of the ATP sulfurylase ranged from 0.23 to 0.25 U/mg.

*Optical Assay for GTP Hydrolysis.* GTP hydrolysis was monitored at 340 nm by coupling the production of GDP to the oxidation of NADH with the coupling enzymes pyruvate kinase and lactate dehydrogenase. The reaction conditions were as follows: NADH (0.11 mM), $MgCl_2$ ([GTP] + [activator] + 1.0 mM), 50 mM HEPES/$K^+$, pH 8.0, $T = 25 \pm 2$ °C. The assays were linear with time and ATP sulfurylase concentration. Less than 10% of GTP was converted to product in all cases.

For reactions involving a single activator, velocities were determined in duplicate at each of 16 conditions obtained using a 4 × 4 matrix of GTP and activator concentrations. In the case of the simultaneous activation by AMP and $PP_i$, two of the three reactants were held fixed at a saturating concentration [$(20-25)K_m$] while the third was varied. For the native reaction, in which GTP hydrolysis is activated by ATP and $SO_4$, the parameters for ATP and GTP were obtained using a 4 × 4 matrix of ATP and GTP concentrations at a saturating concentration of $SO_4$ ($29K_m$); the parameters for $SO_4$ were obtained using a 4 × 4 matrix of $SO_4$ and GTP concentrations at a fixed saturating concentration of ATP ($41K_m$).

The coupling enzymes were desalted using a Superose-12 size-exclusion chromatography column equilibrated with 50 mM HEPES, pH/$K^+$ = 8.0. The coupling enzyme concentrations used in the assays were sufficient to allow the reactions to achieve steady state within 5−10 s, yielding a steady-state GDP level 1−5% that of the total guanine nucleotide concentration (McClure, 1969). The specific activities of pyruvate kinase and lactate dehydrogenase were determined spectrophotometrically in 50 mM HEPES, pH/$K^+$ = 8.0 (Buchler & Pfeiderer, 1955; Dixon & Webb, 1979). The pyruvate kinase specific activity was determined using GDP as the substrate (Davidson, 1959).

*Statistical Analysis of Kinetic Data.* The kinetic parameters were evaluated by fitting the data using the weighted least-squares programs developed by Cleland (1979).

*Radioactive Assay for GTP Hydrolysis.* These assays detect the production of the radiolabeled products [α-$^{32}$P]-GDP or [$^{32}$P]$P_i$ from [α-$^{32}$P]GTP or [γ-$^{32}$P]GTP, respectively. The radiolabeled reactants were separated on 10 cm PEI-F TLC sheets (Randerath & Randerath, 1964) using either a 4.0 M formic acid, pH/$NH_4OH$ = 3.5, mobile phase (Bochner & Ames, 1982), for separation of [α-$^{32}$P]-labeled GDP and GTP, or a 1.0 M LiCl mobile phase, for separation of [$^{32}$P]-$P_i$ and [γ-$^{32}$P]GTP (Randerath & Randerath, 1964). The reactants were quantitated using an AMBIS 2-D radioactivity detector Nye et al., 1988). These assays were used in the pre-steady-state, partition, and isotope trapping experiments depicted in Figures 4, 5, and 6, respectively (see the figure legends for specific conditions).

## RESULTS AND DISCUSSION

*Activation of GTP Hydrolysis.* For a closed system—one that does not exchange mass with its environment—the difference in chemical potential between any two states of the system is independent of the path taken between these two points. The energetic consequence of this First Law of Thermodynamics for allosteric systems is that an increase in affinity for either activator or substrate, that occurs when the second ligand adds to the enzyme, must be accompanied by an identical increase in affinity for the complementary ligand. This energetically equivalent stabilization which occurs simultaneously at both ligand binding sites is the so-called interaction energy (Wyman, 1964; Weber, 1975; Jencks, 1980, 1989). The reciprocal nature of these interactions predicted that the GTP-mediated activation of APS synthesis would have associated effects on the interactions of GTP with the system. The kinetic constants compiled in Table 1 confirm this. The $k_{cat}$ for hydrolysis of GTP ranges from an extremely slow 0.010 $s^{-1}$ to a maximum of 1.8 $s^{-1}$ when activated by AMP. While $k_{cat}$ spans a 180-fold range over the set of activators used, $K_m$ changes very little—the values decrease 2.6−3.6-fold when both activator and substrate are present.

We have previously shown that GTP and ATP bind randomly to the enzyme and that GTP is hydrolyzed in the absence of activators (Leyh & Suo, 1992; Liu et al., 1994). Hence, the allosteric model used in the interpretation of the current results is one in which GTP and the activator bind randomly to the enzyme, and the substrate complexes with and without activator can produce product (see Figure 1). The steady-state rate equations associated with this single

Table 1: Kinetic Parameters for GTP Hydrolysis

| activator/substrate | $K_i$ (μM)[a] | $K_m$ (μM) | $k_{cat}$ ($s^{-1}$) |
|---|---|---|---|
| GTP | | 73 (0.0079)[b] | 0.010 (0.00061) |
| ATP | 150 (13) | 43 (2.1) | 0.18 (0.0024) |
| GTP | 80 (6.3) | 22 (1.2) | |
| $PP_i$ | 45 (3.1) | 17 (0.8) | 0.20 (0.0026) |
| GTP | 71 (5.3) | 27 (1.0) | |
| AMP | 610 (19) | 170 (2.8) | 1.8 (0.0085) |
| GTP | 81 (2.3) | 24 (0.42) | |
| $SO_4$[c] | 80 (6.5) | 42 (2.2) | |
| ATP | 79 (4.6) | 16 (0.65) | 1.3 (0.023) |
| GTP | 31 (1.9) | 6.3 (0.23) | |
| AMP[c] | | 61 (2.6) | |
| $PP_i$ | | 6.4 (0.4) | 1.2 (0.007) |
| GTP | | 4.9 (0.3) | |

[a] $K_i$ is the dissociation constant for the enzyme−ligand complex in the absence of the complementary ligand. [b] The numbers in parentheses indicate the standard error of the data. [c] These are apparent kinetic constants (see Materials and Methods).

[1] Abbreviations: EDTA, ethylenediamine-*N*,*N*,*N*′,*N*′-tetraacetic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; PEP, phosphoenolpyruvate; Tricine, *N*-[*tris*[hydroxymethyl)methyl]glycine; U, unit, micromoles of substrate converted to product per minute at $V_{max}$.

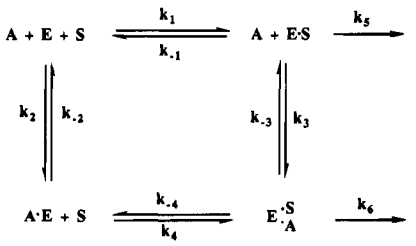


FIGURE 1: Single effector−single substrate activation model. The model shows random binding of substrate, S, and activator, A, to the enzyme. Both the E·S and E·S·A complexes can produce product.

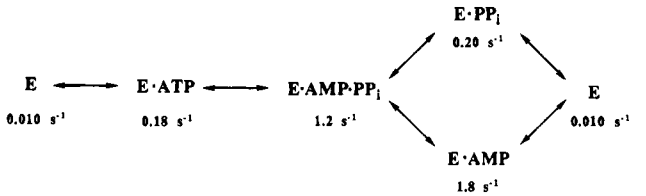


FIGURE 2: Activating complexes that occur in the APS-forming reaction. The progression of activating species in the catalytic cycle for APS synthesis is outlined. Beneath each complex is its $k_{cat}$; the values are from Table 1. The substrate and product guanine nucleotide species associated with each complex were omitted for simplicity.

effector−single substrate model have been derived and discussed in the literature (Frieden, 1964).

*Reaction Coordinate Complexes.* ATP sulfurylase catalyzes hydrolysis of the α,β-bond of ATP in a reaction in which GTP hydrolysis precedes, or is concomitant with, ATP cleavage (Liu et al., 1993). These reactions produce a highly reactive E*AMP intermediate, possibly an enzyme adenylate, which subsequently reacts with sulfate to form APS. The extent to which the individual complexes that form during the native reaction cycle can activate GTP hydrolysis can be studied using ATP, AMP, $PP_i$, or AMP and $PP_i$. These complexes and their associated $k_{cat}$s for GTP hydrolysis are shown in Figure 2. The $k_{cat}$s for the E·AMP and E·AMP·$PP_i$ complexes are 8−10 times those for the E·ATP and E·$PP_i$ complexes, which are nearly identical. Thus, the AMP forms of the enzyme, which presumably mimic the E*AMP intermediate, are the most catalytically active. Moving from the substrate complex, E·ATP, toward the intermediate accelerates completion of the hydrolysis reaction.

*Connectivities between GTP Hydrolysis and Subsections of the ATP Binding Site.* Activation of GTP hydrolysis due to ligand association at the pyrophosphoryl or adenylyl regions of the ATP binding site can be probed separately using AMP or $PP_i$. $k_{cat}$, the maximum number of times a catalytic site can, on average, turn over per unit time, is determined by the structural/energetic disposition of residues at the rate-determining step(s) in the mechanism. Changes in $k_{cat}$ arise either because a different step in the mechanism has become rate-limiting or because the energy/structure of the transition state for the same step has changed. In either case, structural changes underlie rate differences. The nearly identical activation by ATP and $PP_i$ suggests that similar conformational states are induced upon binding of these ligands, and implies that the protein−ligand contacts important for activation occur at the pyrophosphoryl moiety of ATP. The large $k_{cat}$ associated with the E·AMP complex suggests that ATP hydrolysis allows the adenylyl moiety to establish new contacts that provide access to an alternative

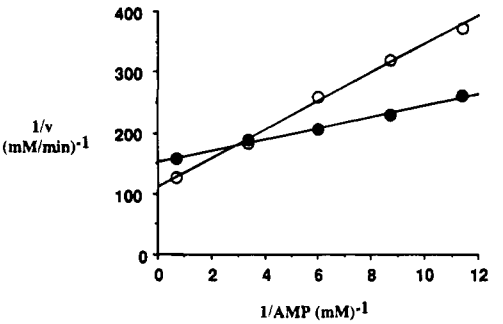


FIGURE 3: Influence of $PP_i$ on AMP activation of GTP hydrolysis. The double-reciprocal plots for AMP activation at zero (○) and a saturating concentration (●) of $PP_i$ are shown. The reaction conditions were as follows: $PP_i$ (0 or 0.50 mM), AMP (at the indicated concentrations), GTP (0.50 mM), $MgCl_2$ ([nucleotide] + [$PP_i$] + 1.0 mM), PEP (0.72 mM), NADH (0.11 mM), ATP sulfurylase (96 nM), pyruvate kinase (3.0 U/mL), HEPES (50 mM, pH/$K^+$ = 8.0), $T = 25 \pm 2$ °C.

set of conformers resulting in a 10-fold increase in $k_{cat}$ over that for the E·ATP complex.

To further investigate the latent activation by the adenylyl moiety of ATP, expressed upon loss of the pyrophosphoryl group, $k_{cat}$ was studied as a function of both AMP and $PP_i$ concentrations. The $K_m$ for AMP and $PP_i$ decreases 2.7-fold when both are present (see Table 1). The influence of a saturating concentration of $PP_i$ on AMP activation is presented in Figure 3. AMP can, depending on its concentration, either activate or inhibit GTP hydrolysis relative to its activation in the absence of $PP_i$. At low AMP concentrations, $K_m$ effects dominate, resulting in an increased velocity; at high AMP concentrations, $K_m$ effects are minimized, $k_{cat}$ effects now dominate, and inhibition is observed. If $PP_i$ and AMP acted independently, their effects on $k_{cat}$ would be additive—$k_{cat}$ would equal 2.0 $s^{-1}$. This is not the case; with both ligands bound, $k_{cat}$ is 1.2 $s^{-1}$, or 0.67 times that predicted on the basis of independent activation. While these experiments do not address the individual contributions of $PP_i$ and AMP to the activation when both are present, they show that $PP_i$ only partially masks the activating potential of AMP compared to that of the pyrophosphoryl moiety on the adenylyl group of ATP. Thus, it appears that formation of the α−β bond of ATP, with its concomitant expulsion of water, inhibits expression of the activating potential of the adenylyl moiety of ATP.

*Hydrolysis of GTP Is Rate-Determining.* Activator-induced changes in $k_{cat}$ are caused by changes in the activation energy(ies) associated with the kinetically significant step(s). To identify the activator-sensitive step(s) in the mechanism, the pre-steady-state production of GDP was studied in the presence and absence of AMP, the most potent $k_{cat}$ effector. A lag or burst of product formation during the first turnover of the enzyme is diagnostic for a slow step(s) preceding or following, respectively, the hydrolytic step(s). A linear progress curve indicates that product release is fast compared to the rate of the chemical event, cleaving the β−γ bond of GTP. While linearity also suggests that assembly of the substrate complexes is fast, it is inconclusive on this point. If GTP adds much more slowly than bond-breaking, a linear progress curve is expected. In the following two sections, we show that this is not the case; both addition and dissociation of GTP are fast.

The pre-steady-state experiments were performed by mixing enzyme, either by hand or by using a rapid mixing

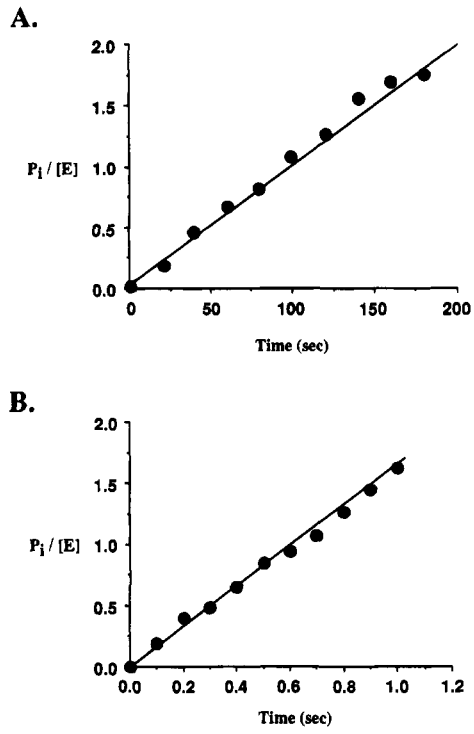


FIGURE 4: Pre-steady-state experiments in the presence and absence of AMP. The progress curves show the formation of $[^{32}P]P_i$ from [$\gamma$-$^{32}$P]GTP versus time. $[^{32}P]P_i$ is expressed in terms of enzyme active site equivalents. Panel A: Hydrolysis in the absence of activator. The reaction was initiated by mixing equal volumes of ATP sulfurylase (80 $\mu$M) and a solution containing [$\gamma$-$^{32}$P]GTP (1.7 mM, SA = 0.04 $\mu$Ci/$\mu$L), $MgCl_2$ (3.7 mM), pyruvate kinase (1.6 U/mL), and PEP (4.0 mM). Panel B: Hydrolysis in the presence of AMP. The reaction was initiated by mixing equal volumes of ATP sulfurylase (40 $\mu$M) and a solution containing [$\gamma$-$^{32}$P]GTP (0.80 mM, SA = 0.04 $\mu$Ci/$\mu$L), AMP (4.0 mM), $MgCl_2$ (6.8 mM), pyruvate kinase (240 U/mL), and PEP (8.0 mM). In the absence of activator, the reactions are slow enough to mix by hand. The experiments that used AMP were mixed with a KinTek quench-flow apparatus. The reactions were performed at 24 ± 2 °C in HEPES (50 mM, pH/$K^+$ = 8.0). Each point is the average of two separate determinations. Reactions were quenched by mixing 1:1 with a solution of 100 mM EDTA, pH/HCl = 9.5. Reactants were separated and quantitated as described under Materials and Methods.

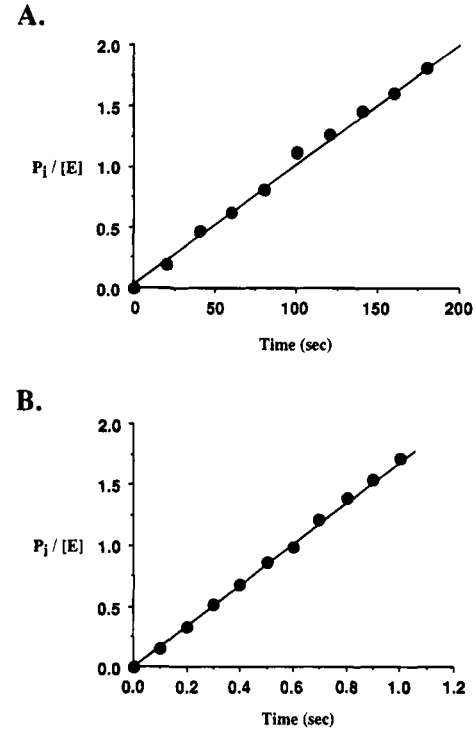


FIGURE 5: GTP partition experiments in the presence and absence of AMP. Panel A: Partitioning in the absence of AMP. The experiment was accomplished by mixing equal volumes of a reacting solution containing ATP sulfurylase (80 $\mu$M), GTP (0.85 mM), $MgCl_2$ (1.85 mM), pyruvate kinase (8.0 U/mL), and PEP (2.0 mM) and an identical chase solution without enzyme containing [$\gamma$-$^{32}$P]GTP (SA = 0.02 $\mu$Ci/$\mu$L). Panel B: Partitioning in the presence of AMP. A solution containing ATP sulfurylase (40 $\mu$M), GTP (0.40 mM), AMP (2.0 mM), $MgCl_2$ (3.4 mM), pyruvate kinase (200 U/mL), and PEP (5.0 mM) was mixed with an equal volume of an identical chase solution without enzyme containing [$\gamma$-$^{32}$P]-GTP (SA = 0.02 $\mu$Ci/$\mu$L). In the absence of activator, the reactions are slow enough to mix by hand using Gilson Pipetteman pipetors. Mixing in the AMP experiments was performed with a KinTek quench-flow apparatus. In both cases, mixing of the first reacting solution was done by hand. The experiments were performed at 24 ± 2 °C in HEPES (50 mM, pH/$K^+$ = 8.0). Each point is the average of two separate determinations. The reactions were quenched by adding 100 mM EDTA, pH/HCl = 9.5, to a final concentration of 50 mM. Reactants were separated and quantitated as described under Materials and Methods.

apparatus, with radiolabeled GTP in the presence and absence of AMP. The conditions of the experiment were such that GTP and activator were near saturating concentration, and the reaction remained pseudo-first-order with respect to enzyme concentration during the measurements which persisted for ~1.8 active site equivalents of product formation. The linear progress curve for GTP hydrolysis, shown in panels A and B of Figure 4, indicates that the hydrolysis rate limits the reaction both in the presence and in the absence of AMP.

*Binding of GTP Is at or near Equilibrium in the Steady State.* The pre-steady-state experiments described above suggest that the reactive complexes assemble quickly compared to product formation; however, they do not directly address the relative rates of hydrolysis and binding of GTP. If association and dissociation are fast compared to cleavage, the concentration of the relevant species are near equilibrium, and the Michaelis constant provides an excellent measure of thermodynamic affinity; that is, $K_m \sim K_d$.

A two-stage mixing experiment was designed to determine if exchange between the enzyme-bound and free pools of GTP is fast compared to product formation. To perform this experiment, enzyme is first mixed by hand with a saturating concentration of unlabeled GTP, in the presence or absence of activator, and a GTP-regenerating system—pyruvate kinase and PEP. This solution is then mixed, either by hand or using a rapid mixing apparatus, with an identical solution containing [$\gamma$-$^{32}$P]GTP and no enzyme. If GTP dissociates slowly compared to its conversion to product, a significant fraction of the enzyme-bound, unlabeled GTP present at the time of mixing with the [$\gamma$-$^{32}$P]GTP solution will be converted to product. This will result in a lag in the formation of labeled product. If GTP dissociation is fast, the dual-mixing and pre-steady-state experiments will yield the same linear progress curve for GTP hydrolysis.

The experimental results, shown in panels A and B of Figure 5, demonstrate linear GDP production throughout the first and second turnover both in the presence and in the absence of activator. These results are, within experimental error, indistinguishable from the pre-steady-state results. The absence of a lag phase indicates that enzyme-bound and solution-phase GTP exchange quickly compared to product formation. Thus, GTP binding is at or near equilibrium, and

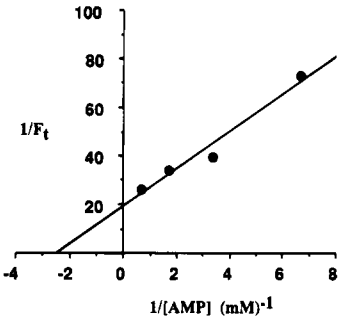


FIGURE 6: Isotope trapping of GTP with AMP. A double-reciprocal plot of the titration of product formation with AMP is shown. The ordinate label, $F_t$, represents the fraction of GTP-bound enzyme active sites converted to product. The pulse solution contained ATP sulfurylase (254 $\mu$M), [$\gamma$-$^{32}$P]GTP (0.50 mM, 0.2 $\mu$Ci/$\mu$L), $MgCl_2$ (1.50 mM), and HEPES (50 mM, pH/$K^+$ = 8.0). Five seconds after adding enzyme to complete the pulse solution, a 17-fold excess volume of chase solution was added. The chase solution contained AMP (at the indicated concentration), GTP (6.0 mM), $MgCl_2$ ([AMP] + [GTP] + 1.0 mM), and HEPES (50 mM, pH/$K^+$ = 8.0). The reaction was then quenched at 2, 3, 4, and 5 s following the addition of chase by adding a 0.40 M stock solution of EDTA, pH 9.5, to a final concentration of 35 mM. Progress curves were constructed for each AMP concentration. The linear least-squares fit to the progress curves was used to extrapolate the fraction trapped back to $t = 0$. The difference between the extrapolated values and a control in which the EDTA was added prior to the chase was used to calculate the fraction trapped. The data shown represent the average of two separate determinations. Reactions were run at 23 ± 2 °C. Reactants were separated and quantitated as described under Material and Methods. The reactions were slow enough to mix the solutions using Gilson Pipettman pipetors.

the $K_m$ for GTP, in the presence and absence of AMP, should very nearly equal the $K_d$ for binding of GTP to E or E•AMP, respectively. These findings confirm that bond-breaking, not addition of GTP, is the rate-limiting step in the reaction.

It was important to ensure that sufficient pyruvate kinase and PEP were added to maintain GTP at or near its initial concentration long enough to conveniently accomplish the second mixing. The duration of the steady state and the concentration of GTP in the steady state were monitored using [$\alpha$-$^{32}$P]GTP. In the absence of AMP, the concentration of GTP at the time of the second mixing was 824 $\mu$M, or 11.3 times its $K_m$ (97 ± 1.0% of its initial value). In the presence of AMP, the concentration of GTP at the time of the second mixing was 344 $\mu$M, or 14.3 times its $K_m$ (86 ± 2.0% of its initial value). AMP was 2.0 mM or 11.8 times its $K_m$. For the exact conditions, see the legend to Figure 5.

*Isotope Trapping of GTP.* To quantitate the relative rates of dissociation and hydrolysis of GTP in the AMP-activated system, an isotope trapping experiment was performed. In this method (Rose, 1980; Cleland, 1975), a pulse solution containing enzyme and radiolabeled GTP is mixed with AMP and a large excess of unlabeled GTP. If GTP is productively bound to the enzyme in the pulse solution and dissociation is sufficiently slow compared to hydrolysis, labeled GDP will form. The fraction of E•GTP that is trapped at an infinite activator concentration gives the relative rates of product formation and desorption of GTP from the E•GTP•AMP complex. The rate constant for dissociation of GTP from the ternary complex can be calculated from these relative rates and $k_{cat}$. Given $K_d$ and the off rate, the rate constant for addition of GTP to E•AMP can be calculated. The result of such an experiment is shown in Figure 6. At an AMP concentration extrapolated to infinity, 5.1% of the E•GTP in the pulse was trapped, indicating that GTP dissociates 19 times faster than it is hydrolyzed; the rate constants for dissociation and association are 35 and 1.4 × $10^6$ $s^{-1}$, respectively.

The isotope trapping and two-stage mixing experiments are complementary in that the traditional trapping method can provide quantitative information even when the rate of dissociation is fast compared to hydrolysis whereas the two-stage experiment, which is not as quantitative when dissociation is fast, allows the full range of intermediates to be sampled. Thus, formation of an intermediate following addition of activator but preceding hydrolysis will be missed by the trapping method if GTP dissociates from the E•GTP complex quickly compared to formation of the intermediate. Such intermediates can be detected by the dual-mixing method.

*Binding of AMP Is at or near Equilibrium in the Steady State.* The initial velocity, steady-state rate equation for an enzyme-catalyzed reaction involving a single substrate (S) and activator (A) has been derived and discussed (Frieden, 1964). The distribution equations are complex; however, careful inspection clearly shows that when substrate binding to E and E•A is at equilibrium, the binding of A to E and E•S must also be at equilibrium. The physical basis for the equivalence of the kinetic, $K_m$, and thermodynamic, $K_d$, constants for AMP binding is easily appreciated if one first considers the case in which GTP is not hydrolyzed— the binding steps, given sufficient time, will equilibrate. If one then allows GTP hydrolysis to occur at a rate that is insignificant compared to adsorption and desorption of GTP, all of the steps preceding hydrolysis will remain essentially at equilibrium. In this case, the double-reciprocal plots represent equilibrium titrations of the enzyme with either activator or substrate, and $K_m$ provides a good approximation of $K_d$ for all of the binding steps.

*Utilization of AMP Binding Energy during GTP Hydrolysis.* The equilibrium binding of GTP and AMP during the steady state allows the system to be represented by a thermodynamic box (see panel A of Figure 7). This box shows that the addition of either GTP or AMP to the enzyme enhances the affinity of the complementary ligand by −0.7 kcal/mol. This modest stabilization occurs only if both the substrate and activator sites are filled; it is the interaction energy in the ground-state E•GTP•AMP, complex.

Another thermodynamic box can be constructed from the available data. This second box, depicted in panel B of Figure 7, links the affinity of the activator for the ground- and transition-state E•GTP complexes to the chemical potential differences between the ground and transition states of the E•GTP and E•GTP•AMP complexes. It is important to realize that the interaction energy for this system, −3.1 kcal/mol, represents the extent to which both the transition state and activator are stabilized. They interact synergistically to produce an enzyme form to which each binds more tightly than it would have in the absence of the other. Thus, as the E•GTP•AMP ground state moves toward its transition state, the affinity for AMP continuously changes in an undefined way to a maximum of −3.1 kcal/mol at the transition state.

The difference in the ground- and transition-state interaction energies demonstrates the enzyme's ability to invoke the chemical potential of its interaction with AMP differently at separate steps in the reaction coordinate. This modulation of the interaction energy allows the enzyme to bring chemical potential to bear as needed in the reaction coordinate to

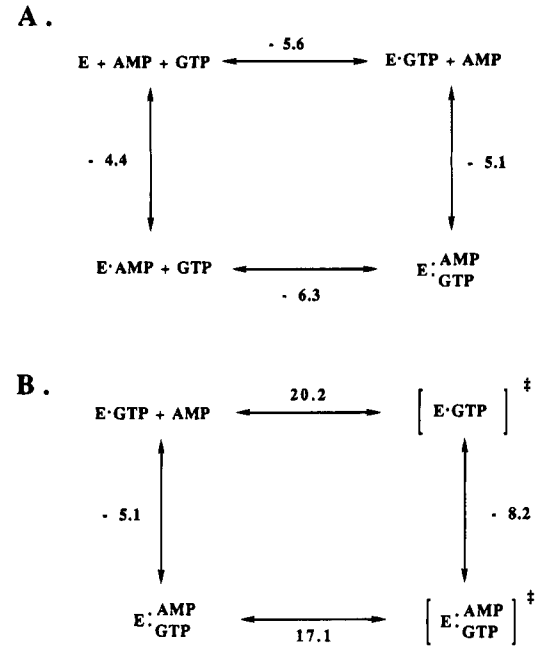


FIGURE 7: Ground- and transition-state stabilization associated with AMP binding. Panel A: Stabilization of ground-state complexes. Panel B: Stabilization of ground- and transition-state complexes. The chemical potentials associated with ligand binding were calculated using a 1.0 M reference state. The transition-state activation energies were calculated at 25 °C using the Eyring equation and assuming a transmission coefficient of unity (Eyring, 1935; Wolfenden, 1976).

accomplish specific chemical tasks. E·GTP binding to ATP results in ground- and transition-state stabilization; as the reaction proceeds to the intermediate E·GTP·AMP complex, the ground-state interaction remains constant, and the transition state undergoes a further, substantial stabilization.

It is interesting that $k_{cat}$ varies considerably, through the activator series; yet, the GTP $K_m$ effects are modest and very similar for all of the activators. This suggests that the structural changes associated with the ground- and transition-state stabilization are, to a certain extent, independent. A plausible model for this independence is that the ground-state stabilization is due to similar interactions with GTP in each case. These protein·GTP contacts may or may not participate directly in the cleavage, possibly occurring at points distal from the cleavage site. Other structural changes, having no apparent effect on $K_m$, result in a unique transition state for each activator. Thus, similar ground-state structures provide a platform from which unique, perhaps subtly different, transition-state structures can be developed by each activator.

## CONCLUSIONS

The ATP sulfurylase catalyzed hydrolysis of GTP is regulated by the substrates for the APS-forming reaction. The binding steps for the addition of GTP and AMP to the enzyme are near-equilibrium. The rate-limiting step in the hydrolysis of GTP, both in the presence and in the absence of AMP, is $\beta,\gamma$-bond cleavage. Transition-state stabilization is substantially greater than that for the ground state; hence, activation is dominated by $k_{cat}$ effects. The ground-state interaction energies remain nearly constant throughout the activator series while those for the transition states vary considerably. This allows the enzyme to employ chemical potential, as needed, at specific points in the reaction coordinate. ATP sulfurylase appears to use this mechanism to link GTP hydrolysis and APS synthesis by coupling formation of the E·AMP intermediate to a substantial increase in the rate of GTP hydrolysis.

## REFERENCES

Bochner, B. R., & Ames, B. N. (1982) *J. Biol. Chem. 257*, 9759−9769.
Bourne, H. R., Sanders D. A., & McCormick, F. (1990) *Nature 348*, 125−132.
Bourne, H. R., Sanders, D. S., & McCormick, F. (1991) *Nature 349*, 117−126.
Buchler, T., & Pfleiderer, G. (1955) *Methods Enzymol. 1*, 435−440.
Cleland, W. W. (1975) *Biochemistry 14*, 3220−3224.
Cleland, W. W. (1979) *Methods Enzymol. 63*, 103−138.
Davidson, E. A. (1959) *Biochim. Biophys. Acta 33*, 238−240.
De Meio, R. M. (1975) in *Metabolic Pathways* (Greenberg, D. M., Ed.) Vol. VII, Academic Press, New York.
Dixon, M., & Webb, E. C. (1979) in *Enzymes*, pp 17−18, Academic Press, New York.
Eyring, H. (1935) *J. Chem. Phys. 3*, 107−115.
Frieden, C. (1964) *J. Biol. Chem. 239*, 3522−3531.
Hopfield, J. J. (1974) *Proc. Natl. Acad. Sci. U.S.A. 71*, 4135−4139.
Imai, Y., Lasky, L. A., & Rosen, S. D. (1993) *Nature 361*, 555−557.
Ishihara, M., Guo, Y., Wei, Z., Zang, Z., Swiedler, S. J., & Orellana, A. (1993) *J. Biol. Chem. 268*, 20091−20095.
Jakoby, W. B., & Ziegler, D. M. (1990) *J. Biol. Chem. 265*, 20715−20718.
Jencks, W. P. (1980) *Adv. Enzymol. Relat. Areas Mol. Biol. 51*, 75−106.
Jencks, W. P. (1989) *Methods Enzymol. 171*, 145−164.
Leyete, A., van Schijndel, H. B., Niehrs, C., Huttner, W. B., Verbeet, M. P., Mertens, K., & van Mourik, J. A. (1991) *J. Biol. Chem. 266*, 740−746.
Leyh, T. S. (1993) *CRC Crit. Rev. Biochem. 28*, 515−542.
Leyh, T. S., & Suo, Y. (1992) *J. Biol. Chem. 267*, 542−545.
Leyh, T. S., Taylor, J. T., & Markham, G. H. (1987) *J. Biol. Chem. 263*, 2409−2416.
Leyh, T. S., Vogt, T. F., & Suo, Y. (1992) *J. Biol. Chem. 267*, 10405−10410.
Lindahl, U., & Hook, M. (1978) *Annu. Biochem. 47*, 358−417.
Liu, C., Martin, E., & Leyh, T. S. (1994) *Biochemistry 33*, 2042−2047.
Liu, C., Suo, Y., & Leyh, T. S. (1994) *Biochemistry 33*, 7309−7314.
McClure, E. R. (1969) *Biochemistry 8*, 2782−2786.
Mudd, H. S. (1980) in *Sulphur in Biochemistry*, pp 239−258, Excerpta Medica, New York.
Mulder, G. J., & Meermen, J. H. N. (1982) in *Sulfate Metabolism and Sulfate Conjugation* (Mulder, G. J., Caldwell, J., Van Kempen, G. M. J., & Vonk, R. J., Eds.) pp 145−153, Taylor Francis, London.
Nye, L., Colclough, J. M., Johnson, B. J., & Harrison, R. M. (1988) *Am. Biotechnol. Lab. 6*, 18−26.
Peck, H. D. J. (1993) in *The Sulfate-Reducing Bacteria: Contemporaary Perspectives* (Odom, J. M., & Singelton, R., Jr., Eds.) pp 41−76, Springer-Verlag, New York.
Randerath, K., & Randerath, E. (1964) *J. Chromatogr. 16*, 111−125.
Rose, I. A. (1980) *Methods Enzymol. 64*, 47−59.
Siegel, L. M. (1975) in *Metabolic Pathways* (Greenberg, D. M., Ed.) Vol. VII, Academic Press, New York.
Weber, G. (1975) *Adv. Protein Chem. 29*, 1−83.
Wolfenden, R. (1976) *Annu. Rev. Biochem. Bioeng. 5*, 271−306.
Wyman, J. J. (1964) *Adv. Protein Chem. 19*, 223−286.

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