Intermediate Channeling between ATP Sulfurylase and Adenosine 5′-Phosphosulfate Kinase from Rat Chondrosarcoma†

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ABSTRACT: Biosynthesis of the activated sulfate donor PAPS (3′-phosphoadenosine 5′-phosphosulfate) involves the sequential action of two enzyme activities. ATP sulfurylase catalyzes the formation of APS (adenosine 5′-phosphosulfate) from ATP and free sulfate, and APS is then phosphorylated by APS kinase to produce PAPS. Using rat chondrosarcoma ATP sulfurylase and APS kinase, a newly developed assay system, which permits measuring the accumulation of both APS and PAPS in the presence of both enzyme activities, produces a PAPS/APS ratio corresponding to a "channeling efficiency" of 96%. The velocity of the APS kinase reaction measured in the overall system with endogenously synthesized APS is 8-fold greater than that of the isolated kinase reaction using exogenous APS. Most conclusively, isotope dilution and enrichment experiments show that the APS intermediate does not equilibrate with APS in the bulk medium but remains largely bound in the rat enzyme system. In contrast, control experiments with a nonchanneled system containing a mixture of the sulfurylase and kinase isolated from *Penicillium chrysogenum* give the results expected for a nonchanneled pathway. These data indicate that APS is channeled between the active sites of ATP sulfurylase and APS kinase during the production of PAPS in rat chondrosarcoma.

PAPS[1] is the high-energy sulfate donor used to generate sulfate esters in mammalian tissues. ATP sulfurylase (ATP, sulfate adenylyltransferase, EC 2.7.7.4) catalyzes the synthesis of APS and pyrophosphate from ATP and free sulfate. APS kinase (ATP, adenylylsulfate 3′-phosphotransferase, EC 2.7.1.25) catalyzes the second step in the sulfate activation pathway which produces PAPS. The APS intermediate produced from ATP and inorganic sulfate is phosphorylated at the 3′-position of the ribose moiety to form the more stable PAPS product.

$$ATP + SO_4^{2-} \rightarrow APS + \text{pyrophosphate}$$

$$APS + ATP \rightarrow ADP + PAPS$$

Since the equilibrium for the ATP sulfurylase reaction is rather unfavorable ($K_{eq} = 10^{-8}$) in the physiologic direction (Farooqui, 1980), APS kinase plays an important role in continually removing the APS, thus driving the overall sulfate activation pathway in the forward direction. PAPS is also structurally more stable than APS, which is subject to spontaneous degradation under physiologic conditions. Although hypotheses have been proposed on the possibility of the coupling of these two enzymes, enzyme titration experiments have indicated that no substrate channeling occurs between the two enzymes from *Penicillium chrysogenum* (Renosto et al., 1989), nor was channeling observed between the enzymes from *Escherichia coli* (Satischandran & Markham, 1989). While these studies suggest that substrate channeling is not a common mechanism in these organisms, a thorough investigation into the nature of PAPS synthesis has not been carried out in mammalian systems. In animals, PAPS serves exclusively as the active sulfate donor, and APS is only an intermediate in the sulfate activation pathway. Furthermore, the cellular level of APS in mammals has been difficult to measure, suggesting that APS might not be released free into solution *in vivo* (Robbins & Lipmann, 1958; Shoyab et al., 1971).

We have pursued an investigation of the mammalian sulfate activation pathway since the genetic defect affecting both ATP sulfurylase and APS kinase was elucidated in the brachymorphic mouse (Sugahara & Schwartz, 1979, 1982). In our subsequent studies of these enzymes from rat chondrosarcoma, ATP sulfurylase and APS kinase were shown to possess similar optimal pH and temperature curves and consistently copurified through gel filtration, ion-exchange chromatography, and affinity chromatography (Geller et al., 1987). These results suggested that there is a physical and functional association of these activities in mammals, and we have recently confirmed that both activities reside on a single, bifunctional protein (Lyle et al., 1994a).

In order to further characterize the interaction between sulfurylase and kinase in more complex organisms, we have undertaken a kinetic examination of sulfate activation. In this study, several approaches were used to demonstrate substrate channeling, such as (a) studying the time course of the appearance of products, (b) calculating the "channeling efficiency" (Paquin et al., 1985), (c) comparing relative velocities, and (d) following the enrichment or dilution of radiolabeled products in the sulfate activation pathway of rat chondrosarcoma.

EXPERIMENTAL PROCEDURES

Materials. The radiolabeled compounds [^{35}S]PAPS (>400 Ci/mmol) and [^{32}P]$Na_4P_2O_7$ (1–20 Ci/mmol) were purchased from New England Nuclear, and [^{35}S]H_2SO_4 (>1000 Ci/mol) was purchased from ICN Radiochemicals. Biogel P-2 and Bio-Rad dye were obtained from Bio-Rad Laboratories. Whatman 3MM Chromatography sheets (46 × 57 cm^2),

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[1] Abbreviations: PAPS, 3′-phosphoadenosine 5′-phosphosulfate; APS, adenosine 5′-phosphosulfate.

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anhydrous pyridine from Aldrich Chemical Co., and ethyl alcohol from Midwest Solvents were used in high-voltage paper electrophoresis. Aqueous Counting Scintillant from Amersham or Universol from ICN was used for scintillation counting. ATP and APS were obtained from Sigma. All other chemicals were reagent grade. The [^{35}S]APS was prepared from [^{35}S]PAPS according to the method of Geller et al. (1987) with yields of 90–95%.

Preparation of Enzymes. The enzyme preparation used in all kinetic assays was purified approximately 2000-fold from rat chondrosarcoma through ammonium sulfate precipitation and chromatography on S-300, hydroxylapatite, and ATP affinity columns as described earlier (Geller et al., 1987). Purified *P. chrysogenum* enzymes (generously provided by Dr. Irwin Segel, University of California at Davis) were stored frozen in 40 mM Tris-HCl, pH 4.0, and were diluted into Buffer A (25 mM $NaHPO_4$–KH_2PO_4 buffer, pH 7.8, 10% glycerol, 1 mM dithiothreitol, 1 mM EDTA) prior to use. *P. chrysogenum* enzymes were diluted to activity levels comparable to the individual sulfurylase and kinase activities found in the rat chondrosarcoma preparation used. For sulfate activation assays, 1.6 nmol of *P. chrysogenum* ATP sulfurylase (0.061 units/mL as measured by molybdolysis assay; Renosto et al., 1990) was combined with 12.4 nmol of *P. chrysogenum* APS kinase (0.21 units/mL as measured by spectrophotometric assay; Renosto et al., 1985). These dilutions of *P. chrysogenum* sulfurylase (K_m(ATP) = 0.13 mM, K_m(SO_4^{2-}) = 0.55 mM, K_m(APS) = 0.3 μM, K_m(PP_i) = 1 μM; Seubert et al., 1985) and the rat chondrosarcoma sulfurylase (K_m(ATP) = 0.2 mM, K_m(SO_4^{2-}) = 97 μM, K_m(APS) = 39 μM, K_m(PP_i) = 18 μM; Lyle et al., 1994b) both contained an activity of 0.334 nmol/min as measured by the reverse sulfurylase assay described below. The *P. chrysogenum* kinase (K_m(ATP) = 1.5 mM, K_m(APS) = 1.4 μM; Renosto et al., 1984) and the rat chondrosarcoma kinase (K_m(ATP) = 24 μM, K_m(APS) = 76 nM; Lyle et al., 1994c) each exhibited an activity of 0.013 pmol/min as measured by the kinase assay described below at 200 μM ATP and 40 nM [^{35}S]APS. Protein concentrations were determined according to the Bio-Rad assay protocol with bovine γ-globulin as the standard and Bio-Rad dye as the stain.

Enzyme Assays. The ATP sulfurylase assay used is a modification of that described previously (Geller et al., 1987) which measures the velocity of the reaction in the direction of ATP formation. A standard 1.0-mL incubation mixture contained the following components at the indicated final concentrations: 50 mM $NaHPO_4$–KH_2PO_4 buffer (pH 7.8), 12 mM $MgCl_2$; 0.5 mM dithiothreitol; and 5 mM NaF, 0.2 mM $Na_4P_2O_7$ (containing 6.66 μCi of $^{32}PP_i$), and 0.1 mM APS, from a 5 mM stock kept in 50% ethanol at −20 °C to minimize decomposition.

The assay for APS kinase was that previously described (Geller et al., 1987). The 25-μL reaction mixture contained 20 mM $MgCl_2$, 4 μM [^{35}S]APS, 10 mM ATP, and 12 μL of enzyme preparation. For studies comparing the kinase to the sulfate activation assay, the concentration of APS was adjusted accordingly.

The sulfate activation assay was a modification of a procedure described by Sugahara and Schwartz (1982). The standard 25-μL reaction mixture contained 0.4 mM [^{35}S]H_2SO_4, 10 mM ATP, 20 mM $MgCl_2$, 22 mM Tris-HCl (pH 8.0), and 10 μL of enzyme preparation. Added pyrophosphatase had a slightly negative effect on overall activity (data not shown) and thus was not included in the reaction mixture. Products were separated and measured as described previously (Geller et al., 1987).

Isotope Dilution and Enrichment. Isotope dilution was analyzed by two methods. In the first, unlabeled APS at varying concentrations was added to the overall reaction mixture (with ATP at 2 mM and $^{35}SO_4^{2-}$ at 0.5 mM), and the reaction was started by adding the enzyme. For the other isotope dilution and enrichment experiments, the reaction was started as above, but the unlabeled APS was added after 4 min, or the reaction was started as above with unlabeled SO_4^{2-}, and [^{35}S]APS was then added. The reaction was stopped by addition of ice-cold ethanol and freezing on dry ice. Products were separated and measured as described previously (Geller et al., 1987).

For some experiments, a second method for separating and quantitating nucleotide products was developed based on a modification of the method of Lee et al. (1984). Briefly, 50 μL of the reaction mixture was separated by reverse-phase ion-paired HPLC on a Jones Chromatography APEX Octadecyl 5 μm column (50-cm × 4.6-mm i.d.). The mobile phase (15 mM tetrabutyl ammonium hydroxide, 100 mM ammonium acetate, and 10% methanol, adjusted to pH 7 with acetic acid) was run at a flow rate of 1 mL/min. The nucleotide peaks were detected at 254 nm and quantitated by comparison with the peak areas of standard concentrations. Radioactivity (^{35}S) was measured by scintillation counting.

RESULTS

Overall Reaction. In order to probe the interaction between ATP sulfurylase and APS kinase, the activities were evaluated in the context of an overall reaction system. When ATP and radiolabeled sulfate were incubated with an enzyme preparation containing both sulfurylase and kinase activities, the amounts of labeled APS and PAPS produced were measured simultaneously. A time course for the production of both APS and PAPS using this approach is presented in Figure 1. PAPS appeared without any detectable lag period for the accumulation of the intermediate APS, and its production was linear through the first 12 min. APS production reached a steady-state concentration (60 nM) within 2 min and remained constant throughout the time course. This assay allowed for the determination of APS intermediate channeling by various methods.

Isotope Dilution. The traditional and most conclusive method of defining substrate channeling, the use of isotope dilution or enrichment, was used to study the sulfate activation pathway. In one representative set of experiments, unlabeled APS was included with the ATP and labeled sulfate, and the reaction was initiated by addition of enzyme. In this case, the starting concentration of endogenous labeled APS was zero. The pattern of labeled PAPS production is illustrated in Figure 2. The point at which 50% of the PAPS is labeled corresponds to approximately 3.8 μM unlabeled exogenously added APS, while the endogenous steady-state concentration of labeled APS was measured at 63.5 ± 5.2 nM. Thus, the concentration of exogenous unlabeled APS which results in 50% isotope dilution is 60-fold higher than the steady-state level of endogenous APS. However, although the steady-state level of APS appears to be constant from as early as 2 min, the starting concentration is zero, so the ratio of exogenous to endogenous APS is much greater at the beginning of the reaction. Therefore, the isotope dilution experiments were also carried out by adding unlabeled APS at 4 min after initiation of the reaction, when the steady-state level of endogenous APS had been achieved. A range of concentrations of unlabeled APS was added to the sulfate activation assay initiated with ATP and labeled sulfate. Only a small decrease in the production of labeled PAPS was observed at all concentrations of added APS. For example, at a 10 000-

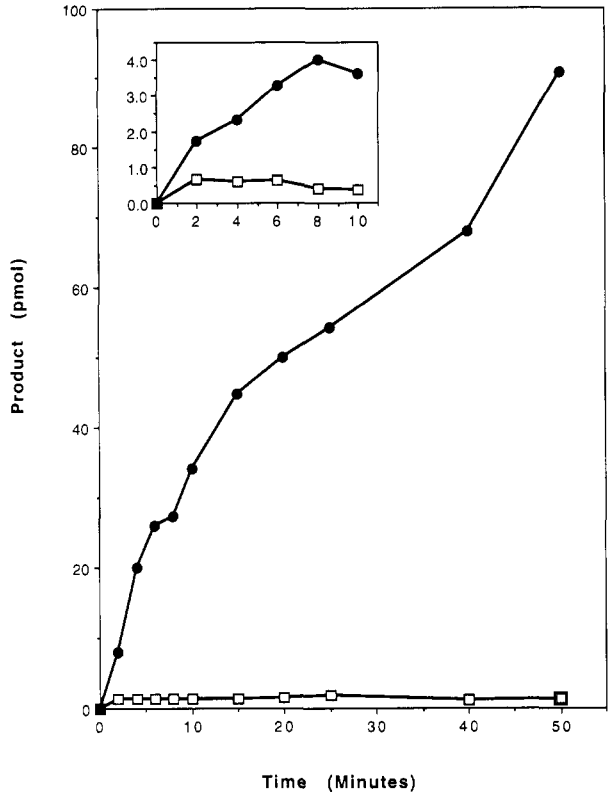

FIGURE 1: Time course of rat chondrosarcoma sulfate activation assay. Both PAPS (●) and APS (□) were measured simultaneously. Substrate concentrations were $^{35}SO_4^{2-}$ 0.4 mM, >1000 Ci/mol; ATP 10 mM; and $MgCl_2$ 20 mM. Inset: time course of *P. chrysogenum* enzymes. Specific activities for reverse sulfurylase (0.334 nmol/min) and kinase (0.013 pmol/min) were the same for the rat and fungal assays.

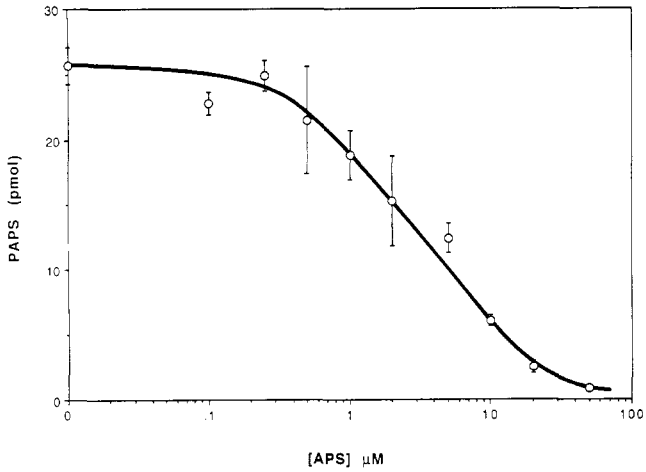

FIGURE 2: Isotope dilution of rat chondrosarcoma sulfate activation assay. The production of [^{35}S]PAPS by the sulfate activation assay (containing 0.578 nmol/min ATP sulfurylase, 0.0352 pmol/min APS kinase, 0.5 mM $^{35}SO_4^{2-}$, 2 mM ATP, and 20 mM $MgCl_2$) was measured at various concentrations of unlabeled APS (prepared fresh in 50 mM Tris-HCl, pH 8.0). The reaction time was 10 min.

fold higher level of unlabeled APS, only a 26% reduction of labeled PAPS production was obtained (Table 1).

In further experiments, isotopically labeled APS was added to the sulfate activation assay (initiated with unlabeled substrates) at a concentration approximately 2 times the steady-state concentration of endogenously produced APS. In this case, less than 10% of the synthesized PAPS was labeled. Clearly, the APS intermediate is not released free into solution, where it would be diluted or enriched by exogenously added APS, but rather it is concentrated at the kinase active site.

Table 1

	Isotope Dilution steady-state [APS][a]	[APS] added	dilution[b] (%)
rat chondrosarcoma	50 nM	500 μM	26
P. chrysogenum	14 nM	25 nM	67
	Isotope Enrichment steady-state [APS][c]	[APS] added	enrichment (%)
rat chondrosarcoma	85 nM	143 nM	9.5
P. chrysogenum	22 nM	143 nM	615

[a] Observed concentrations of [^{35}S]APS when exogenous APS was added to a reaction containing 0.334 nmol/min sulfurylase, 0.013 nmol/min kinase, 2 mM ATP, 0.5 mM SO_4^{2-}, and 20 mM $MgCl_2$. The APS was added at 4 min, and the total reaction time was 10 min. [b] Percent decrease in observed level of [^{35}S]PAPS formed compared to control. Rat PAPS, 53 843 (control), 39 973 cpm (diluted); fungal PAPS, 6628 (control), 2223 cpm (diluted). [c] Control assay initiated with $^{35}SO_4^{2-}$ used to measure APS concentration.

Table 2: Effect of Exogenous APS on PAPS Production[a]

[APS] (μM)	total PAPS[b]	labeled PAPS[c]	labeled APS[c]
0.0	25	25	2
0.2	32	29	2
0.5	36	37	3
1.0	37	37	4
2.0	50	32	8
5.0	42	9	13
10.0	44	9	18
20.0	34	5	27

[a] Observed production of APS and PAPS when exogenous APS, at the listed concentrations, was added to a reaction containing 1.24 nmol/min sulfurylase, 0.062 pmol/min kinase, 2 mM ATP, 0.5 mM SO_4^{2-}, and 20 mM $MgCl_2$. The reaction time was 10 min. [b] Total PAPS produced was determined by UV absorbance at 254 nm following separation from reactants by HPLC. All quantities are in pmol. [c] [^{35}S]PAPS and [^{35}S]APS were measured by liquid scintillation counting of UV-identified peaks. All quantities are in pmol.

Table 1 presents examples of these isotope dilution and enrichment experiments.

To confirm that the decrease in labeled PAPS is not associated with a decrease in total PAPS synthesis, a series of isotope dilution experiments was designed in which total PAPS formed was determined. In these experiments, total PAPS was measured by HPLC analysis. As shown in Table 2, there was little variation in total PAPS over the entire exogenous APS concentration range. In addition, the ratio of labeled PAPS to total PAPS decreases significantly only at exogenous APS concentrations greater than 2 μM while the steady-state level of endogenous labeled APS was approximately 40 nM. Concomitant with this, a significant increase in the steady-state level of labeled APS was observed only at concentrations of exogenous APS above 2 μM. Thus, these data indicate that a decrease in PAPS specific activity occurs only at exogenous APS concentrations high enough to enter the pathway, uncouple the channeling mechanism, and cause a release of the endogenous APS.

In contrast to rat chondrosarcoma, when the *P. chrysogenum* enzyme system was analyzed using isotope dilution and enrichment experiments, it was clear that no channeling existed between the activities (Table 1). Unlabeled APS, at a concentration only 2-fold greater than the steady-state level, added to the sulfate activation assay initiated with labeled sulfate, resulted in a 67% reduction in the amount of labeled PAPS formed. This is the expected amount of reduction in a system where complete mixing of the intermediate with the

Table 3: Initial Velocities of PAPS Formation[a]

	overall reaction	kinase assay	ratio (overall:kinase)
rat chondrosarcoma	3.40	0.43	8.0:1
P. chrysogenum	0.87	0.24	3.6:1

[a] Initial velocities are given in pmol/min. The steady-state levels of APS produced by the overall reaction (containing 0.4 mM SO_4^{2-}), 10 mM ATP, 20 mM $MgCl_2$, 0.334 nmol/min sulfurylase, and 0.013 pmol/min kinase) were 60 (rat preparation) and 25 nM (fungal preparation). These were the respective APS concentrations used in the individual kinase reactions in order to obtain the ratios of PAPS formation at this APS level in the overall system and in the individual kinase assay.

bulk solution occurs. Isotope enrichment with labeled APS showed the characteristics of complete mixing as well. When a 6.6-fold higher concentration of labeled APS was added to the sulfate activation assay started with unlabeled substrates, 6.2-fold more labeled PAPS was produced. Thus, the labeled exogenous APS has the same access to the kinase active site as the APS produced by the sulfurylase activity. Therefore, *P. chrysogenum* exhibits the results expected for a system where channeling behavior does not pertain.

Channeling Efficiency. The time course of the sulfate activation assay allowed for the calculation of the "channeling efficiency" (Paquin et al., 1985). The ratio initial rate of PAPS production/initial rate of APS production (APS + PAPS measured) corresponds to a 96% efficiency of channeling. Thus, almost all of the initial APS produced was directly converted to PAPS through the channeling mechanism. Similar studies were conducted with a sample containing a mixture of purified ATP sulfurylase and APS kinase from *P. chrysogenum*, which have been shown to act independently of each other with no channeling (Renosto et al., 1989). Figure 1 (inset) represents a time course of the sulfate activation assay performed on a sample containing both sulfurylase and kinase from *P. chrysogenum* (see Methods). Although there did not appear to be a lag in the appearance of PAPS, the velocity and overall level of PAPS production were much lower than in the rat chondrosarcoma system, and the steady-state concentration of APS was about one-half the value observed in the rat system. The calculated channeling efficiency of the fungal enzymes (~80%) was also lower than that of the rat enzymes.

Kinase Velocity. The channeling phenomenon can also be described by comparing the initial velocity of the kinase reaction alone to the velocity of PAPS production in the overall system (Table 3). As mentioned, the steady-state APS concentration obtained in the time course with the enzymes from rat chondrosarcoma was 60 nM. This concentration was used to measure the initial velocity (nmol/min) of the kinase reaction alone, starting with ATP and labeled APS. The initial velocity of the kinase reaction by this method was 8-fold lower than the initial velocity of PAPS production measured by the sulfate activation assay. Thus, the kinase enzyme was more efficient when APS supplied by the sulfurylase enzyme was used than when APS was provided exogenously. A comparison of the initial velocity for the kinase reaction alone to the velocity in the sulfate activation assay also produced different results for the fungal enzymes. In this case, the steady-state concentration of APS observed in the sulfate activation assay of the fungal preparation was 25 nM. This concentration was used to measure the initial velocity of the kinase reaction alone. The kinase velocity in the sulfate activation assay was 3.6-fold greater than the initial velocity of the kinase reaction alone (Table 3). Although these results are significantly different from those obtained with the rat chondrosarcoma enzymes, they do suggest that coupling

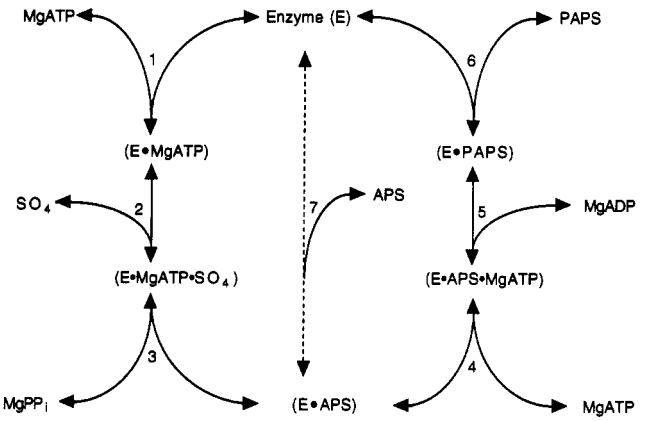

FIGURE 3: Mechanism of the sulfate activation pathway. ATP sulfurylase and APS kinase act functionally as a three substrate, three product enzyme.

benefits these sequential reactions in *Penicillium* preparations.

Overall Sulfate Activation. The demonstration of APS intermediate channeling establishes that the two activities are kinetically linked. In addition, it has been determined that the individual reaction mechanism for ATP sulfurylase from rat chondrosarcoma is sequential, ordered with ATP as the first substrate to bind in the physiologic direction and APS as the first substrate to bind in the reverse direction (Lyle et al., 1994b). The mechanism of the individual APS kinase reaction is also sequential, ordered with APS as the first substrate and PAPS as the second product released (Lyle et al., 1994c). Thus, their interaction might be described in the functional context of overall sulfate activation as the pathway shown in Figure 3. In this mechanism, the ATP sulfurylase and APS kinase would act functionally as a three substrate, three product enzyme, and the rate equation in double reciprocal terms would be

$$\frac{E_0}{V_0} = \frac{1}{k_{+1}(\mathrm{ATP})} + \frac{k_{-1}(k_{-2}+k_{+3})}{k_{+1}k_{+2}k_{+3}(\mathrm{ATP})(\mathrm{SO_4})} + \frac{k_{-2}+k_{+3}}{k_{+2}k_{+3}(\mathrm{SO_4})} + \frac{k_{-4}+k_{+5}}{k_{+4}k_{+5}(\mathrm{ATP})} + \frac{1}{k_{+3}} + \frac{1}{k_{+5}} + \frac{1}{k_{+6}}$$

This equation consists of a conventional ordered, "ternary complex" mechanism (Cornish-Bowden, 1979) involving ATP as the leading substrate and sulfate as the following substrate with the usual (ATP) and (SO_4) terms and also the expected (ATP)(SO_4) cross term. The second entry of ATP is kinetically isolated from the entries of sulfate and the earlier ATP and thus creates the second (ATP) term but no cross term with SO_4 or ATP (which would give parabolic double reciprocal plots for ATP if it were present). The equation assumes that the APS intermediate remains complexed with the enzyme, and thus the rate constant for APS release (+7 of Figure 3) is insignificant. This mechanism should generate a linear double reciprocal initial velocity plot with respect to ATP concentration. The sulfate activation assay was used to test the proposed mechanism and resulting equation by measuring the synthesis of the final product, PAPS, at various concentrations of ATP. Kinetic data were processed by unweighted iterative nonlinear least-squares fitting of initial velocity as a function of substrate concentration. The kinetic parameters obtained by fitting the data points to a rectangular hyperbolic plot were then used to graph the initial velocity pattern in linear double reciprocal form as shown in Figure 4. The initial velocity pattern presented in Figure 4 supports

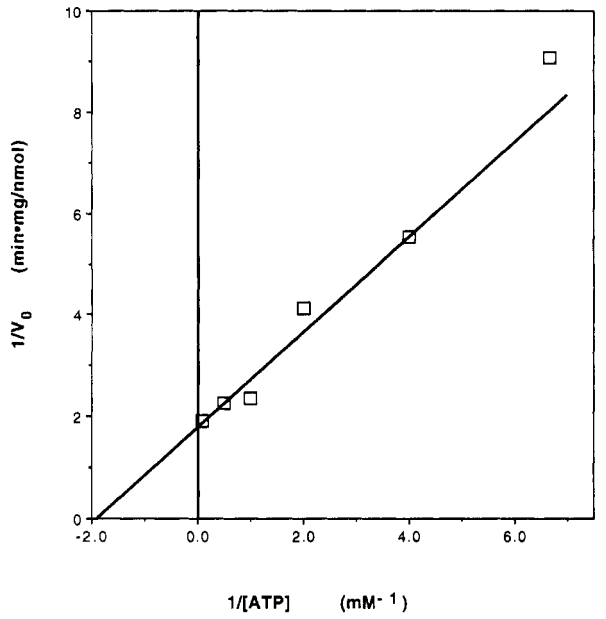

FIGURE 4: Production of PAPS by the sulfate activation assay of rat chondrosarcoma. The production of [^{35}S]PAPS by the sulfate activation assay (containing 1.28 nmol/min ATP sulfurylase, 0.078 pmol/min APS kinase, 0.25 mM $^{35}SO_4^{2-}$, and 20 mM $MgCl_2$) was measured at various concentrations of ATP. Kinetic data were processed by iterative nonlinear least-squares fitting of initial velocities as a function of ATP concentration. The resulting parameters were used to derive the double reciprocal line shown.

the rate equation and the proposed ternary complex mechanism.

DISCUSSION

We have had a long-standing interest in the sulfate activation pathway since the unique defect was elucidated in the brachymorphic mouse which affects both ATP sulfurylase and APS kinase (Sugahara & Schwartz, 1979, 1982). Because previous studies designed to characterize the sulfurylase and kinase enzymes from rat chondrosarcoma showed that these two activities copurified (Geller et al., 1987), it became obvious that the structural and functional relationships between these two enzyme activities must be elucidated. In order to gain a better understanding of the interactions of the sulfate activating enzymes, we have undertaken an analysis of the functional cooperation in the overall pathway.

The phenomenon of substrate channeling, which represents the transfer of an intermediate between the active sites of two or more enzyme activities without equilibrating with the bulk solution, has been demonstrated in a number of systems. Paquin et al. (1985) described the lack of a lag time in the appearance of the second product in a two enzyme pathway. They also calculated the "channeling efficiency" from the ratio of the initial rate of appearance of the final product/initial rate of intermediate production. Intermediate channeling has been well characterized for tryptophan synthase, for which the crystal structure is known (Hyde et al., 1988; Anderson et al., 1991). Several groups (Srivastana et al., 1989; Fahien et al., 1989; Fukushima et al., 1989) have used enzyme titration to describe the channeling phenomenon. This method relies on the failure of E_1 to inhibit E_2 based on the predicted ratio of $[E_1{\cdot}S]/[S_{free}]$. Although this method requires the accurate experimental determination of the dissociation constant of S for E_1, it does provide compelling evidence of direct transfer when combined with independent proof of either stable or transient complex formation between the enzymes. However, while this method may be effective for separable enzymes such as those in the glycolytic pathway, it is not feasible for stable multifunctional complexes or bifunctional enzymes. These systems are more amenable to the strategy employed by Belkaid et al. (1988), who utilized isotope dilution experiments to demonstrate that the intermediate did not mix with exogenously added substrate in the pyrimidine pathway. Because enzyme titration experiments have shown that channeling does not occur in the sulfate activation pathway of *P. chrysogenum*, where the enzymes are separable (Renosto, et al., 1989), several other methods were used to explore this possibility in rat chondrosarcoma, where the activities have been inseparable. These methods were also applied to a preparation containing both *P. chrysogenum* activities for comparison. One potential problem with this comparison could be caused by PAPS acting as an allosteric inhibitor, as has been shown for fungal sulfurylase (Renosto et al., 1990). However, the $[I]_{0.50}$ was found to be $\sim$35 μM in *P. chrysogenum*, while the concentration of PAPS produced in the sulfate activation assays of our control studies never reached 300 nM. Furthermore, we have no evidence that PAPS acts as an allosteric inhibitor of rat chondrosarcoma ATP sulfurylase. Therefore, this effect should not be a factor in the comparison of the two systems by these methods.

In a channeled system without leakage, the intermediate does not mix with the bulk solvents. Therefore, a labeled intermediate will not be diluted by exogenously added unlabeled substrate in an assay initiated with labeled substrates. The *P. chrysogenum* enzymes exhibit the results expected when complete mixing of the intermediate and the bulk solution occurs and thus no channeling exists. In contrast, the rat system does not allow free APS to readily enter the pathway. Assuming that the steady-state level of endogenous APS is constant throughout the reaction, the concentration of exogenous, unlabeled APS needed to give 50% dilution is at least 60-fold higher than the level of endogenous APS produced by the sulfurylase enzyme. Thus, if the endogenous and exogenous APS concentrations were to be equal, one exogenous APS molecule would enter the pathway for every 60 molecules of endogenous APS molecules passing through the system. Further isotope dilution and enrichment experiments were performed in which the exogenous APS was added at 4 min of the reaction, after the steady-state level of APS had been achieved. In this case, there was considerably less dilution even at much higher exogenous APS concentrations. These experiments clearly show that exogenous APS does not have access to the kinase active site comparable to that of endogenous APS formed from ATP and sulfate.

The quantitation of both labeled and unlabeled PAPS production shows that there is no decrease in total PAPS synthesis in the presence of exogenous APS. In addition, the reduction of the specific activity of PAPS occurs only when the exogenous APS concentration is 30–50-fold in excess of the steady-state APS generated by the enzyme. Thus, a high concentration of APS is required to uncouple the channeling mechanism and cause significant isotope dilution. These data also indicate that added APS, even at high concentrations, does not act as an inhibitor of ATP sulfurylase. All of these types of analyses are definitive for the phenomenon of channeling, and the results show unequivocally that APS produced by the sulfurylase activity is not released free into solution, where it can mix with the bulk solvent, but is channeled to the active site of the APS kinase.

The other approaches used are also consistent with APS intermediate channeling in the sulfate activation pathway of rat chondrosarcoma and help illustrate the channeling phenomenon in conjunction with the isotope dilution data. However, taken by themselves, these types of analyses may

have other explanations and therefore must always be considered more fully. For instance, the channeling efficiency calculations indicate a significant conversion of the APS intermediate to the final product PAPS. Although these data suggest substrate channeling, it could be argued that the extremely low K_m(APS) and the high catalytic activity of the kinase enzyme are the sole causes of the low APS steady-state concentration and the lack of a measurable lag time for the appearance of PAPS. Thus, the system could be exhibiting very efficient coupling. Indeed, the two isolated *P. chrysogenum* enzymes when added together displayed approximately 80% efficiency, which is significant but not as high as the 96% efficiency demonstrated by the inseparable rat chondrosarcoma activities. In addition, the comparison of the kinase velocity in the overall reaction to the kinase reaction alone lends more support to a channeled pathway. The kinase enzyme appears to function more efficiently when supplied with APS from the sulfurylase enzyme than from the bulk solution. However, in a multienzyme complex or a bifunctional protein with no channeling, a conformational change caused by complex formation or by the catalytic activity of the first reaction might cause the K_m(APS) for the overall system to be much lower than that of the kinase reaction alone (Brooks & Storey, 1988). In the nonchanneled *P. chrysogenum* system, the overall kinase activity was 3.6-fold higher than the kinase activity alone, suggesting that some cooperation might exist in this system. However, this result is not commensurate with the more extensive enzyme titration studies (Renosto et al., 1989), which clearly showed that sulfurylase-bound APS does not serve as a substrate for *P. chrysogenum* kinase. The assay conditions used here to optimize the rat enzymes may have a different effect on the fungal enzymes, favoring the fungal sulfurylase activity over the kinase. As seen in Tables 1 and 3, the endogenous concentration of APS differs between the rat and fungal systems. The steady-state level of APS is most likely determined by the interaction of the two activities intrinsic to each system. Since APS is channeled by the rat enzyme and mostly not released free into solution, the steady-state APS measured in the rat system may represents largely enzyme-bound APS. In sum, the results of these experiments in rat chondrosarcoma help to describe the channeling phenomenon more fully and confirm the results of the conventional isotope dilution and enrichment experiments.

The kinetic behavior presented in Figure 4 supports the rate equation and the mechanism illustrated in Figure 3. The deviation of points which appear in the linear double reciprocal plot are not represented as significantly poor fits in untransformed hyperbolic plots. Although the pathway of PAPS synthesis is not obligated to follow this mechanism in order to achieve intermediate channeling, the specific order of substrate addition and product release appears to be the most effective means of accomplishing this goal. As shown, APS can bind the free enzyme, thus allowing for the study of the individual kinase and reverse sulfurylase reactions. However, as demonstrated by the isotope dilution experiments, the access of free APS to the active site is minimal in the context of the overall channeled system.

Srere (1987) offers several explanations for the existence of channeling including the calculation that 80% of metabolic intermediates have only one use in the cell. Certain aspects of the sulfate activation pathway provide instances where intermediate channeling could supply a distinct advantage. The equilibrium constant for the sulfurylase reaction strongly favors the physiologic reverse direction. Direct transfer of APS achieves higher substrate concentrations at the kinase active site with fewer APS molecules. In addition, the APS intermediate is chemically (spontaneous degradation) as well as biochemically (degradation by sulfohydrolases) labile, and channeling is a mechanism which aids in its preservation. Although the activities need not reside on the same polypeptide to achieve intermediate channeling, the rat chondrosarcoma activities may also have this added advantage, as previously suggested (Geller et al., 1987) and confirmed by recent evidence (Lyle et al., 1994a). The change to a channeled system in the rat chondrosarcoma (relative to the fungal system) appears to be an example of a pathway which has become more efficient in order to overcome the inherent obstacles to the synthesis of the universal high-energy sulfate donor, PAPS.

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