Rat Chondrosarcoma ATP Sulfurylase and Adenosine 5′-Phosphosulfate Kinase Reside on a Single Bifunctional Protein†

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ABSTRACT: The sulfate-activation pathway consists of the sequential action of ATP sulfurylase (ATP: sulfate adenylyltransferase, EC 2.7.7.4) and adenosine 5′-phosphosulfate kinase (ATP:adenylylsulfate 3′-phosphotransferase, EC 2.7.1.25). Both sulfurylase and kinase from rat chondrosarcoma were copurified through substrate affinity chromatography using stable analogs of APS (adenosine 5′-phosphosulfate) and PAPS (3′-phosphoadenosine 5′-phosphosulfate). A 56-kDa protein, containing both activities, was then purified to apparent homogeneity through reversed-phase chromatography and observed on denaturing polyacrylamide gels. The molecular mass of the native active unit containing both activities in the purified preparation corresponded to approximately 60 kDa by analytical gel filtration. Coincident binding and elution of ATP sulfurylase and APS kinase by immunoaffinity chromatography, using polyclonal serum generated against the 56-kDa protein, also demonstrated that the enzyme contains both activities. Lastly, a single N-terminal amino acid sequence was obtained from the 56-kDa band isolated by gel electrophoresis. These results all suggest that ATP sulfurylase and APS kinase from rat chondrosarcoma reside on a single bifunctional protein.

We have had a long-standing interest in the sulfate-activation pathway since elucidating the unique defect in the brachymorphic mouse which affects both ATP sulfurylase and APS[1] 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 relationship between these two enzyme activities must be elucidated. In order to gain a better understanding of the physical association of the sulfate-activating enzymes, it was necessary to purify the activities.

Both ATP sulfurylase and APS kinase are required for the activation of inorganic sulfate to its high-energy form, PAPS. However, the purification of each activity from a single species has only been reported for the fungus *Penicillium chrysogenum* (Seubert et al., 1980; Renosto et al., 1984) and *Escherichia coli* (Leyh et al., 1988; Satischandran & Markham, 1989). ATP sulfurylase has been purified from a number of lower organisms (Renosto et al., 1990; Onajobi et al., 1973; Nozawa, 1980; Renosto et al., 1991). The enzyme generally varies in size from 42 to 67 kDa and may form oligomers. In addition to the fungal and bacterial sources, APS kinase has been isolated from the alga *Chlamydomonas reinhardii* (Jender & Schwenn, 1984). The kinases of these lower organisms are smaller proteins, 21–44 kDa, which form dimers.

There are only a few reports of the isolation of these enzymes from higher organisms. The purification of mammalian ATP sulfurylase has been described for rat liver. Burnell and Roy (1978) reported a M_r = 410 kDa, but the enzyme was not homogeneous on acrylamide gel electrophoresis. Yu et al. (1989) published a native size of 122 ± 12 kDa, composed of 62 ± 6 kDa subunits; however, the most purified preparation contained significant "contaminating" APS kinase activity. Matsuo et al. (1987) showed that rat liver sulfurylase and kinase elute together at approximately 68 kDa on gel filtration chromatography. However, large molecular weight aggregates containing enzyme activity were also eluted under certain conditions. Hommes et al. (1987) have reported that rat liver APS kinase is a 58-kDa protein comprised of four equal subunits. In earlier studies, rat chondrosarcoma ATP sulfurylase and APS kinase copurified over 2000-fold through S300 gel filtration, hydroxylapatite, and ATP-affinity chromatography (Geller et al., 1987), suggesting that the activities are inseparable. Both activities from rat chondrosarcoma chromatographed together at an estimated size of 260 kDa under nondenaturing conditions (Geller et al., 1987). SDS–PAGE revealed three bands at 35–37, 60, and 150 kDa, suggesting that the native enzyme may be a complex of these smaller proteins. An alternative interpretation is that only one of the proteins contains both activities and that the other bands represent contaminants, breakdown products, or aggregates of the partially purified protein. Data presented in this manuscript are consistent with the latter interpretation.

Clearly, there are physical differences between the enzymes of mammals and those of lower organisms. In addition, there are some differences among the results reported for the enzymes of mammalian sources. Therefore, these purification studies, which elucidate the nature of the interaction between ATP sulfurylase and APS kinase in rat chondrosarcoma, also help explain the distinction between mammals and lower organisms and clarify earlier studies of this important pathway in higher organisms.

EXPERIMENTAL PROCEDURES

Materials. The radiolabeled compounds, [^{35}S]PAPS (>100 Ci/mmol) and [^{32}P]$Na_4P_2O_7$ (1–20 Ci/mmol), were pur-

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[1] Abbreviations: APS, adenosine 5′-phosphosulfate; PAPS, 3′-phosphoadenosine 5′-phosphosulfate; Me-APS, adenosine 5β-[(methylenephospho)sulfate]; Me-PAPS, 3′-phosphoadenosine 5β-[(methylenephospho)sulfate]; TFA, trifluoroacetic acid.

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chased from New England Nuclear, and [^{35}S]H_2SO_4 (>1000 Ci/mol) was obtained from ICN Radiochemicals. The ATP-affinity column contains adenosine 5′-triphosphate linked to agarose through an 11-atom spacer and was purchased from Pharmacia. The adipic acid hydrazide used in the synthesis of the substrate analog affinity columns was also purchased from Pharmacia. Protein A affinity disks were purchased from Amicon. Problott PVDF membranes were obtained from Applied Biosystems. ATP, APS, CNBr-activated Sepharose-4B, and bovine serum albumin were purchased from Sigma Chemicals. Whatman 3MM chromatography sheets (46 × 57 cm), anhydrous pyridine from Aldrich Chemical Co., and ethyl alcohol from Midwest Solvents were used in high-voltage paper electrophoresis. Aqueous counting scintillant from Amersham was used for scintillation counting. All other chemicals were reagent grade. The Me-APS and Me-PAPS were synthesized in this laboratory as previously described (Callahan et al., 1989; Ng et al., 1991). The [^{35}S]APS was prepared from [^{35}S]PAPS as outlined (Geller et al., 1987).

Preparation of Enzymes. The enzyme preparation was purified from rat chondrosarcoma through ammonium sulfate precipitation and chromatography on S-300, hydroxylapatite, and ATP-affinity columns as described earlier (Geller et al., 1987). Although the enzyme assay protocols, including the specific activities of labeled substrates, differed slightly from those previously outlined, these purification steps were generally comparable to those reported earlier (Geller et al., 1987). 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 has been previously described (Geller et al., 1987). The APS kinase assay was modified from that earlier outlined (Geller et al., 1987). The 25-μL reaction mixture contained 20 mM $MgCl_2$, 100 mM ammonium sulfate, 100 nM [^{35}S]APS (>100 Ci/mmol), 10 mM ATP, and 15 μL of enzyme preparation. The overall assay, measuring the production of APS and PAPS simultaneously, 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. The mixture reacted for 10 min at 37 °C and was stopped by the addition of ice-cold ethanol and freezing on dry ice. Products were separated and measured as described previously (Geller et al., 1987).

Me-APS- and Me-PAPS-Affinity Chromatography. Me-APS was coupled to agarose–adipic acid hydrazide through its ribose hydroxyl groups as outlined (Callahan et al., 1989). Me-PAPS was coupled to Sepharose 4B as previously described (Ng et al., 1991). The column matrix was diluted into buffer A (25 mM $NaHPO_4$–KH_2PO_4 buffer, pH 7.8, 10% glycerol, 1 mM dithiothreitol, 1 mM EDTA) and poured into the column. The matrix was washed with 100 mL of buffer A and the nonspecific sites were blocked with 50 mL of bovine serum albumin (1 mg/mL in buffer A). The column was washed sequentially with 100 mL of buffer A, 100 mL of 1 M KCl in buffer A, and 100 mL of buffer A. Hydroxylapatite-purified enzyme (50 mL) was loaded and the flow through was reloaded. The column was washed with 50 mL of buffer A and eluted with a stepwise gradient of 0.05 M KCl, 0.25 M KCl, and 0.5 M KCl in buffer A. Fractions of 5.0 mL were collected and assayed for sulfurylase, kinase, and overall activity. Active fractions were concentrated and dialyzed into buffer A by passing through Amicon CF 25 Centriflo membrane cones. The pool of active fractions was concentrated to ~100 μL in Centricon 30 ultrafiltration concentrators.

Antibody Generation and Analysis. The ATP-affinity-purified sulfurylase/kinase complex of rat chondrosarcoma was separated by SDS–PAGE. The 56-kDa band was then excised from the gel and injected into rabbits (New Zealand white) for the production of polyclonal antibodies (Harlow & Lane, 1988). The immunoreactivity of the serum was tested by ELISA and Western blot. The rabbit serum was extracted and the antibodies were purified over a protein A disk. The purified polyclonal antibodies were coupled to CNBr-activated Sepharose-4B to prepare antibody affinity columns.

Amino Acid Analysis and Sequencing. ATP-purified rat chondrosarcoma sulfurylase/kinase enzyme was run in three lanes on 10% PAGE and transferred to PVDF using the Applied Biosystems protocol. The transfer time was 75 min at a constant 170 mA using a Bio-Rad plate-electrode electrophoresis tank. The blot was stained with coomasie blue and the 56-kDa band of each lane was excised. The blots were washed extensively with deionized water and stored frozen. One lane was subject to amino acid analysis by immersing the blot in 20 μL of 20% phenol (v/v) and 200 μL of 6 M HCl. The protein was hydrolyzed and coupled to PITC as described previously (Heinrickson & Meredith, 1984). The sample was analyzed on an octadecyl column (4.5 × 250 mm) to determine amino acid composition and to quantify the amount of protein present in the sample. The other two lanes were analyzed for N-terminal amino acid sequence data. The PVDF membranes were trimmed to the appropriate size and were loaded into the Applied Biosystems 473A pulse-liquid sequencer.

RESULTS AND DISCUSSION

In order to show that the sulfurylase and kinase enzymes are separable, form a multifunctional complex, or reside on a bifunctional polypeptide, the purification to apparent homogeneity was attempted. The partial purification of ATP sulfurylase and APS kinase from rat chondrosarcomas has been previously described (Geller et al., 1987). All preparations used for further analysis were purified through the hydroxylapatite or ATP-affinity steps and represent an enrichment of approximately 80- or 2000-fold, respectively, as earlier outlined (Geller et al., 1987). Both activities consistently copurified with no disruption in the overall sulfate-activation through these early steps (data not shown). The purification was monitored by using the sulfurylase and kinase assays as well as the new overall assay, which is initiated with ATP and free sulfate and measures the production of both APS and PAPS simultaneously. A greater enrichment of one activity with respect to the other would result in a disruption of the relative levels of APS and PAPS measured by this assay.

Column Chromatography. The first separation approach presented here was based on affinities for different substrates and products of the enzymes. The substrate analogs of APS and PAPS were synthesized in this laboratory (Callahan et al., 1989; Ng et al., 1991) in order to complete the kinetic analysis of the PAPS synthesizing enzymes and were shown to act as inhibitors, competitive with respect to the substrates APS and PAPS (Lyle et al., 1994 a,b). Importantly, Me-APS and Me-PAPS are stable ligands which can be coupled to gel matrices to prepare affinity purification systems. Rat chondrosarcoma sulfurylase/kinase enzyme preparations, purified through the hydroxylapatite step, were loaded onto

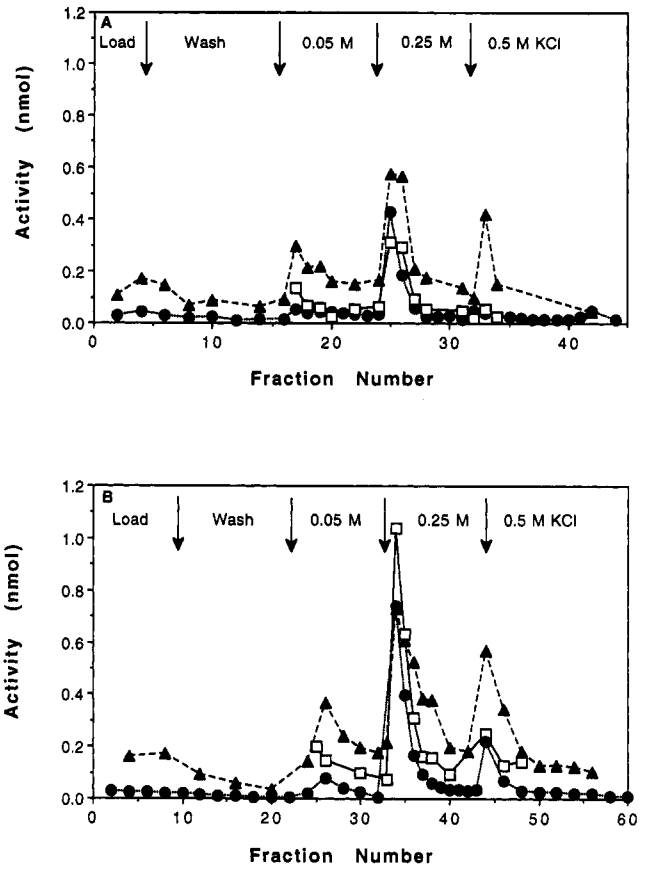

FIGURE 1: Me-APS and Me-PAPS-affinity chromatography. The hydroxylapatite-purified rat chondrosarcoma enzyme (Geller et al., 19897) was diluted into 50 mL of buffer A and loaded onto the Me-APS (A) or Me-PAPS (B) column. The activities, (●) ATP sulfurylase (nmol of ATP/10), (▲) APS kinase (nmol of PAPS × 10^4), and (□) overall activity (nmol PAPS × 100) were eluted with a stepwise gradient of KCl in buffer A. The main peaks of activity were dialyzed in buffer A for comparison to the column load.

Table 1: Purification of ATP Sulfurylase and APS Kinase through Affinity Chromatography

			ATP sulfurylase		APS kinase	
column fraction	volume[a]	total protein[b]	specific activity[c]	total activity[d]	specific activity[e]	total activity[f]
Me-APS load	40	3.6	28	101	3.6	13
Me-APS elution	10	0.082	300	25	37	3.0
Me-PAPS load	45	4.2	25	104	3.4	14.5
Me-PAPS elution	28	0.15	420	62	53	7.8

[a] Volume in mL. [b] Total protein in mg. [c] Sulfurylase specific activity in nmol of ATP/min·mg. [d] Total sulfurylase activity in nmol of ATP/min. [e] Kinase specific activity in pmol of PAPS/min·mg. [^{35}S]APS was ~500 Ci/mmol. [f] Total kinase activity in pmol/min.

an Me-APS-affinity column and eluted with a stepwise gradient of increasing salt concentration. As shown in Figure 1A, both activities as well as the overall sulfate-activation activity copurify with a peak in the 0.25 M KCl elution. Enzyme that was bound nonspecifically to the matrix was eluted in 0.05 M KCl and residual activity that remained bound after 0.25 M KCl was eluted with 0.5 M KCl. Preparations were also loaded onto a Me-PAPS column and the activities again coelute with the major peak in the 0.25 M KCl step (Figure 1B). The enzyme preparation was purified over 10-fold through the Me-APS column and ~17-fold through the Me-PAPS column (Table 1). Although there are minor peaks eluting from the columns in which the ratio of sulfurylase to kinase activity varies, the two enzyme active sites likely respond to the micro environment in different ways. Thus while both activities reside on the same protein, each activity will not necessarily remain constant under all conditions.

More importantly, it can be concluded that both affinity matrices exhibit specificity for the sulfate-activating enzymes. It was not unexpected that both ATP sulfurylase and APS kinase would bind and coelute from the Me-APS-affinity column, since APS is the common intermediate linking the reactions, i.e. the product of the sulfurylase and the substrate for the kinase. However, since PAPS is the product of only the kinase reaction, it is not readily apparent why ATP sulfurylase should bind to the Me-PAPS column, unless the sulfurylase is bound as a consequence of its association with the kinase. Furthermore, while PAPS and Me-PAPS inhibit the kinase reaction, they do not inhibit rat chondrosarcoma ATP sulfurylase (Lyle et al., unpublished results). Thus, it appears that the ATP sulfurylase does not contain a PAPS allosteric effector site, as is the case with *P. chrysogenum* sulfurylase (Renosto et al., 1990). Moreover, the sulfurylase activity is unlikely to have low affinity for PAPS, which allows it to bind. In this case, the kinase activity would become enriched in comparison to the sulfurylase in the elution from the Me-PAPS column. However when the purification steps were also monitored by the new overall assay, in which incorporation of labeled sulfate into both APS and PAPS is measured simultaneously, there was no difference in the relative levels of APS and PAPS. Therefore, it appears that rat chondrosarcoma ATP sulfurylase is physically associated with APS kinase.

Although indicative of a direct association of the two activities, copurification through the affinity columns does not differentiate between a single bifunctional enzyme or an interactive complex of multiple protein species. Therefore, the enzymes were examined under conditions designed to dissociate and separate proteins that may be interacting in a potential enzyme complex. Toward this end, purification to apparent homogeneity was attempted by reversed-phase chromatography. The highly purified ATP-affinity preparations (Geller et al., 1987) were passed through a C_4 reversed-phase HPLC column. The sample was loaded in HPLC-grade H_2O with 0.1% TFA and eluted with a gradient from H_2O (0.1% TFA) to 70% acetonitrile (0.1% TFA), 30% H_2O (0.1% TFA) as shown in Figure 2. Fractions were collected, dialyzed, and concentrated in buffer A and then assayed for activity. Three major protein peaks (1, 2, and 7 in Figure 2) were routinely eluted, with peak 2 containing approximately 37% of the total protein eluted on the basis of UV absorbance at 280 nm. Both ATP sulfurylase and APS kinase activities elute together within peak 2, and the material contained in these fractions exhibited a single 56-kDa protein band on SDS–PAGE (Figure 3). Several minor protein peaks (3–6 in Figure 2), containing sulfurylase and kinase activity, also eluted from the C_4 column. However, these peaks had lower specific activity and purity than the major peak and all together accounted for less than 20% of the total activity recovered from the column. The specific activity of the major peak represented an increase of 1.4-fold (kinase) and 1.3-fold (sulfurylase) over the highly enriched ATP-affinity-purified preparation (Table 2). The low yield (14%) of activity recovered through this column represents the loss of enzyme activity under the harsh conditions needed to elute the protein from the column. Recovery of enzyme activity was dependent upon immediate dialysis into the nondenaturing buffer A and concentration of the dilute fractions. The disruption of native enzyme structure caused by the elution solvent had to be

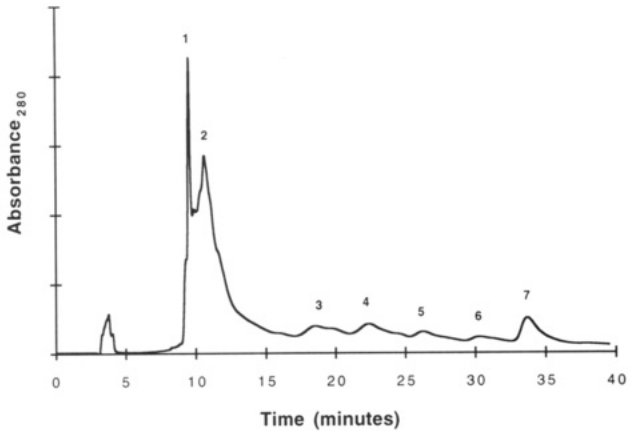

FIGURE 2: Elution of C_4 reversed-phase column. The C_4 reversed-phase column was loaded with 100 μL of ATP-affinity purified rat chondrosarcoma ATP sulfurylase/APS kinase in buffer A, washed with H_2O (0.1% TFA) for 6 min, and eluted with a gradient from 100% H_2O (0.1% TFA) to 30% H_2O (0.1% TFA) 70% acetonitrile (0.1% TFA). The elution was monitored by absorbance at 280 nm. Peaks 1 (9–10 min), 2 (11–15 min), 3 (17–21 min), 4 (22–25 min), 5 (26–28 min), 6 (29–32 min), and 7 (33–36 min) were pooled, dialyzed in buffer A, concentrated, and assayed for activity. Peak 2 contained 80% of the enzyme activity recovered with the remaining 20% in peaks 3–6. The ratios of sulfurylase:kinase activity in all active peaks were comparable.

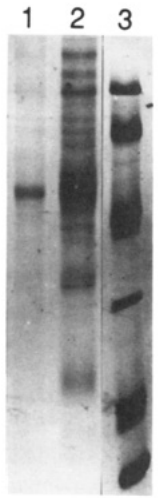

FIGURE 3: Purification of the ATP sulfurylase/APS kinase complex as shown on 10% SDS–PAGE. Lane 1, C_4 reversed-phase column elution (7.7 μg of protein); Lane 2, C_4 column load (47.6 μg of ATP-affinity purified enzyme preparation); Lane 3, molecular weight markers, phosphorylase b (97 400 Da), bovine serum albumin (66 200 Da), ovalbumin (42 699 Da), carbonic anhydrase (31 000 Da), soybean trypsin inhibitor (21 500 Da), and lysozyme (14 400 Da). The gel was stained with coomassie and silver in combination.

reversed before enzyme activity could be measured. Thus, the sulfurylase/kinase enzyme system appears to consist of the single polypeptide of 56 kDa as observed by gel electrophoresis, although the active species may consist of oligomers of this single protein.

In order to determine whether the 56-kDa band seen on SDS–PAGE is the active unit or whether a higher order of association pertains, the purified enzyme was applied to an analytical TSK-3000 gel filtration column with buffer A as the solvent. When the column was standardized with known molecular weight markers, the peak in sulfurylase and kinase activities corresponded to a molecular mass of 60 kDa (Figure 4), which is consistent with the 56 kDa determined by denaturing gel electrophoresis. Thus, the single 56-kDa protein appears to be the minimal active unit which contains both

Table 2: Purification of ATP Sulfurylase and APS Kinase through C_4 Reversed-Phase Chromatography

				ATP sulfurylase		APS kinase	
fraction	volume[a]	protein concn[b]	total protein[c]	specific activity[d]	total activity[e]	specific activity[f]	total activity[g]
load	100	3.4	340	380	130	42	14.2
elution	200	0.17	34	500	17	59	2.0

[a] Volume in mL. [b] Protein concentration in mg/mL. [c] Total protein in mg. [d] Sulfurylase specific activity in nmol of ATP/min·mg. [e] Total sulfurylase activity in nmol of ATP/min. [f] Kinase specific activity in pmol of PAPS/min·mg. [^{35}S]APS was ~54 Ci/mmol. [g] Total kinase activity in pmol/min.

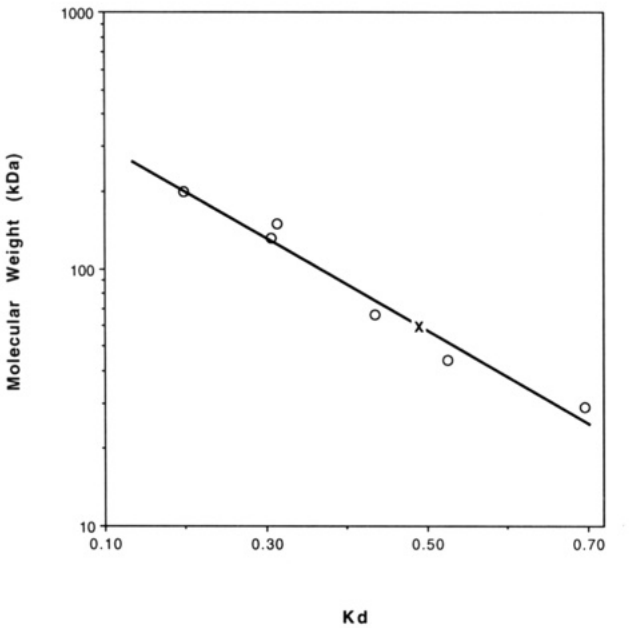

FIGURE 4: Analytical gel filtration chromatography of purified ATP sulfurylase/APS kinase complex. C_4 column purified enzyme in buffer A was run on an analytical TSK-3000 gel filtration HPLC column. ATP sulfurylase and APS kinase activities, as well as the overall activity, eluted together as a peak marked X, corresponding to a native molecular mass of 60 kDa. Molecular weight markers were β-amylase (200 000 Da), alcohol dehydrogenase (150 000 Da), bovine serum albumin dimer (132 400 Da), bovine serum albumin monomer (66 200 Da), ovalbumin (42 699 Da), and carbonic anhydrase (31 000 Da).

enzyme activities and does not need to form larger oligomeric complexes for activity. However, it is still possible that under certain conditions the enzyme forms aggregates, as previously described (Geller et al., 1987; Matsuo et al., 1987; Hommes et al., 1987), which may or may not represent the physiologically relevant state.

Antibody Analysis. In order to ascertain whether the two activities reside on separable proteins which are not resolved by SDS–PAGE or HPLC, antibody reagents were used to examine the enzyme preparation. Polyclonal serum was generated against the 56-kDa protein isolated from rat chondrosarcoma for use in immunoaffinity chromatography. The polyclonal antibody affinity column bound both ATP sulfurylase and APS kinase, quantitatively, until saturation of both activities was achieved (Figure 5). The loss of kinase activity, which has been shown to be more labile (Geller et al., 1987), may account for the difference in the activity levels. Both activities were eluted together in 1.5 M glycine, 3.0 M NaCl, pH 10.0, with recovery of the 56-kDa protein as detected by PAGE (Figure 6). However, less than 10% of the activity bound to the matrix was recovered due to the harshness of the elution conditions. Several alternative elution buffers con-

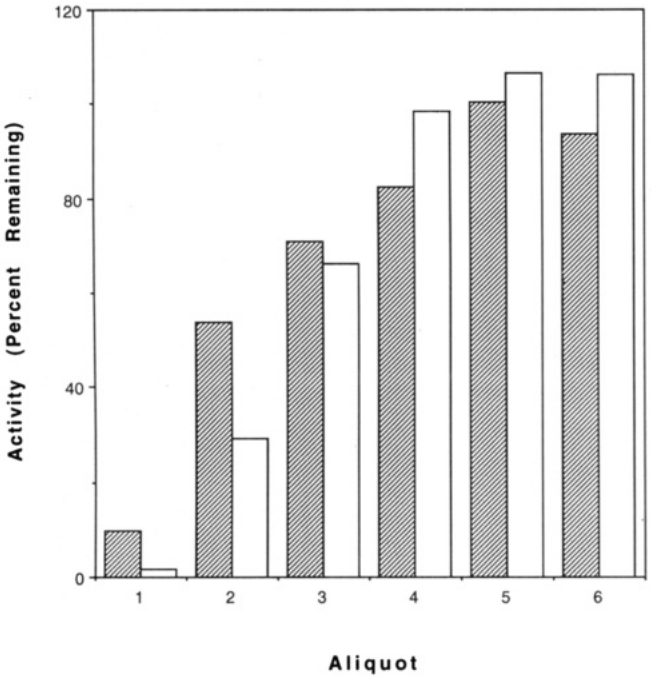

FIGURE 5: ATP sulfurylase/APS kinase saturation of a polyclonal antibody column. ATP affinity purified enzyme (Geller et al., 1987) was diluted into 10 mM Na_2HPO_4–KH_2PO_4, 0.15 M NaCl, pH 7.2, and several aliquots of equal volume were passed through the polyclonal antibody affinity column and collected separately. Each aliquot was assayed for ATP sulfurylase (hatched) and APS kinase (open) activity. The percent activity was calculated as the total activity present in the preloaded aliquot compared to the activity which passed through the column.

taining glycine and NaCl at various pH were attempted, but each was deleterious to the enzyme. Although confirming the coelution of both activities, the immunoaffinity column was not useful as a purification step due to the substantial loss of enzyme activity during elution. However, the specificity exhibited by the polyclonal sera demonstrates that this immunologic reagent is a valuable probe for the ATP sulfurylase/APS kinase enzyme for use in other studies such as Western blot analysis and cDNA library screening. More importantly, these studies indicate, by an independent means, that the antibody reacts with a single peptide that contains both activities.

N-Terminal Sequencing. In order to confirm that the 56-kDa band observed on PAGE represented only one protein and to obtain amino acid sequence data for rat chondrosarcoma ATP sulfurylase/APS kinase, N-terminal sequencing was performed on ATP-purified enzyme preparation. This preparation contains high enzyme activity and allows good separation of the 56-kDa band from other proteins by SDS–PAGE (see Figure 3, lane 2). The enzyme preparation was loaded into three lanes, separated by SDS–PAGE, and transferred to PVDF. The 56-kDa bands were excised and one band of the sulfurylase/kinase enzyme was subjected to amino acid analysis to determine the amount of enzyme present. A single band was estimated to contain 500 pmol of protein; thus, the remaining two protein bands (approximately 1 nmol of enzyme) were analyzed by pulse-liquid amino acid sequencing using the Edman degradation technique. A single N-terminal sequence was found and determined to be Glu-Val-Gln-Leu-Val-Glu-Ser-Gly-GLy-Gly-Leu-Val-Gln-Pro. The peaks of the residues eluted from the sequencer were approximately equal to 440-pmol amounts, which represent a 44% yield; this is comparable to the yield obtained with standard protein (BLG) from PVDF membranes. The 14-

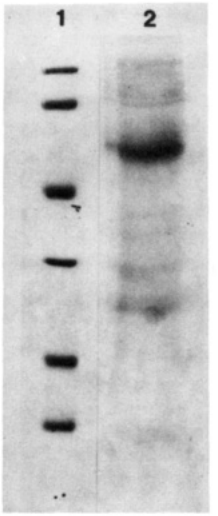

FIGURE 6: 10% SDS–PAGE of polyclonal immunoaffinity-purified sulfurylase/kinase. Lane 1, molecular weight markers, phosphorylase b (97 400 Da), bovine serum albumin (66 200 Da), ovalbumin (42 699 Da), carbonic anhydrase (31 000 Da), soybean trypsin inhibitor (21 500 Da), lysozyme (14 400 Da); Lane 2, immunoaffinity purified rat chondrosarcoma ATP sulfurylase/APS kinase (~5.7 μg of protein). The gel was stained with coomassie and silver in combination.

residue N-terminal sequence obtained for rat chondrosarcoma ATP sulfurylase/APS kinase was examined against the major sequence databases and was found to be closely related to the first 14 residues of Ig heavy chain V-region. Although the sequence similarity was greater than chance, no conclusions can be made regarding the significances of this similarity based on a short sequence. An examination of analogous sequences can be done when the sequencing of the enzyme by molecular techniques is complete.

As mentioned, the enzymes involved in sulfate activation that have been described in lower organisms all appear to be relatively small and contained on separate proteins. In fact, three gene products are necessary for sulfate-activation in *E. coli* (Leyh et al., 1988), while only two are required in yeast and various fungi (Seubert et al., 1980; Renosto et al., 1984). It is an intriguing possibility that the 56-kDa bifunctional protein from rat chondrosarcoma may represent the fusion of genes similar to those found in lower organisms. This change to a bifunctional protein may be an example of an evolutionary modification of this pathway in higher organisms. Whereas coordinate expression can be obtained in procaryotes by linking different polypeptides in a single operon, linking multiple functions in a single polypeptide is a more common mechanism for coordinate expression in eucaryotes. In fact, multifunctional polypeptides have been described for a number of enzyme activities (de Mata & Rabinowitz, 1980; Rouzer et al., 1986; Schrimsher et al., 1986). In addition, it has been proposed that certain bifunctional enzymes such as 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase represent the gene fusion of catalytic units (Bazan et al., 1989). Also, the bifunctional peroxisomal enoyl-CoA hydratase/3-hydroxyacyl-CoA dehydrogenase has sequence similarity to the separate mitochondrial enoyl-CoA hydratase and 3-hydroxyacyl-CoA dehydrogenase, suggesting that the enzymes have common evolutionary origins (Minami-Ishii et al., 1989). Hartl (1989) has provided interesting theories about protein evolution from small functional units, some of which may pertain to mammalian sulfurylase/kinase. The formation of the rat chondrosarcoma bifunctional enzyme may have followed such a general combinatorial process in order to achieve maximal

functional efficiency. Genetic sequence data might offer some clues about how this process may have occurred.

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