Sulfate-Activating Enzymes in Normal and Brachymorphic Mice: Evidence for a Channeling Defect†

Stephen Lyle, Jeffrey D. Stanczak, John Westley, and Nancy B. Schwartz*

Departments of Pediatrics and Biochemistry and Molecular Biology, University of Chicago, Chicago, Illinois 60637

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ABSTRACT: The severe reduction in the amount of 3′-phosphoadenosine 5′-phosphosulfate (PAPS) in cartilage from homozygous brachymorphic mice results from a decrease in the activities of both ATP sulfurylase (50%) and adenosine 5′-phosphosulfate (APS) kinase (14% of normal). In order to better understand the etiology of this double enzyme defect, a dual approach to elucidating the nature of the enzyme complex as well as its mechanistic properties was undertaken. Antibody reagents that react with both activities provide evidence for a single, bifunctional protein in both normal and mutant cartilage. Quantitative Western blot analysis indicates that a normal amount of a dysfunctional protein is produced in mutant cartilage. Kinetic studies show that the V_{max} for mutant kinase is significantly reduced and that mutant sulfurylase and kinase appear to have lower K_m^{APS} values than normal. Interestingly, the mutation appears to disrupt the channeling mechanism that has recently been demonstrated for this pathway [Lyle et al. (1994) *Biochemistry 33*, 6822−6827]. APS kinase from normal mouse cartilage utilizes APS supplied by ATP sulfurylase much more efficiently than APS which is added exogenously; i.e., channeling efficiency is >90%. In contrast, the mutant enzymes exhibit only 54% channeling efficiency. Lastly, isotope dilution and enrichment experiments show directly that the APS binding sites of the mutant enzymes are more accessible to free APS than are those of the normal enzymes. These data suggest that the mutation primarily affects the catalytic properties of the PAPS activation system by altering the function of the novel coupling mechanism between the two activities, causing a decrease in the ability to channel APS and produce PAPS efficiently.

Brachymorphism in mice is characterized by shortened limbs, tail, and snout (Lane & Dickie, 1968). Homozygous recessive brachymorphic (bm/bm) mouse cartilage contains normal amounts and types of collagen and glycosaminoglycans. However, the glycosaminoglycans of mutant tissue are undersulfated, which causes the proteoglycans to form smaller aggregates (Orkin et al., 1976, 1977). These biochemical abnormalities are strikingly manifested in the growth zones of long bones in the mutant mice; the columnar zone has normal organization but is about 50% of the normal size while the hypertrophic zone is less than 30% of normal (Orkin et al., 1976). Incorporation of labeled sulfate into APS,[1] PAPS, and chondroitin sulfate of cell-free mutant extracts is much lower than in normal cartilage extracts, and the specific activities of ATP sulfurylase (ATP:sulfate adenylyltransferase, EC 2.7.7.4) and APS kinase (ATP: adenylylsulfate 3′-phosphotransferase, EC 2.7.1.25) are reduced to 50% and 14% of normal levels, respectively (Schwartz et al., 1978; Sugahara & Schwartz, 1979). The enzyme activities from mutant cartilage are more cold-labile than normal, and heterozygous cartilage exhibits intermediate levels of enzyme activity for both sulfurylase and kinase (Sugahara & Schwartz, 1982). These results indicate that the mutation affects both enzymes of the sulfate activation pathway. More recent experiments have been designed to elucidate the nature of the association between these two activities, thereby beginning to understand the dual enzyme defect in the brachymorphic mouse.

ATP sulfurylase and APS kinase from rat chondrosarcoma were earlier shown to possess similar pH and temperature optima curves (Geller et al., 1987) and now have been demonstrated to be physically linked in a single, bifunctional protein (Lyle et al., 1994b). Antibody reagents generated against the rat chondrosarcoma enzyme have been used to probe the activities in normal and brachymorphic mouse cartilage preparations in the present study.

Rat chondrosarcoma ATP sulfurylase and APS kinase have also been shown to be functionally linked through the mechanism of intermediate channeling (Lyle et al, 1994a). Several approaches were used to describe this phenomenon which clearly show that the intermediate APS generated by the ATP sulfurylase is not released free into solution but is concentrated at the active site of APS kinase. Using the information derived from these mechanistic studies, we have now undertaken kinetic analysis of ATP sulfurylase and APS kinase in normal and brachymorphic mouse cartilage. The results strongly suggest that the integrity of the APS channeling mechanism of the brachymorphic enzymes is affected by the mutation.

MATERIALS AND METHODS

Materials. The radiolabeled compounds, [^{35}S]PAPS (>400 Ci/mmol), [^{32}P]$Na_4P_2O_7$ (1−20 Ci/mmol), and [^{35}S]H_2SO_4

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* Author to whom correspondence should be addressed, at the Department of Pediatrics, University of Chicago, MC5058, 5841 S. Maryland Ave., Chicago, IL 60637. Tel: 312-702-6426. Fax: 312-702-9234.

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

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(>1000 Ci/mol), were purchased from New England Nuclear, and all other chemical reagents used for these studies were of the highest quality as previously described (Geller et al., 1987). The anti-ATP sulfurylase/APS kinase monoclonal antibody was isolated in this laboratory (see below). The second antibody was goat anti-mouse IgG, and the third antibody was mouse peroxidase/anti-peroxidase (Sigma Chemicals).

Preparation of Enzymes. Cartilage tissue was obtained from age-matched 3−5 day old normal (C57BL/6J) and homozygous brachymorphic (bm/bm) mice. The distal femoral heads and proximal tibial heads were dissected according to Sugahara and Schwartz (1982). The cartilage tissue was homogenized on ice in buffer A (0.025M NaH_2PO_4 and K_2HPO_4, pH 7.8, 1 mM DTT, 1 mM EDTA, and 10% glycerol), sonified, and centrifuged at 9000*g* for 30 min, at 4 °C. Aliquots of the supernatant were stored at −20 °C for further studies. Preparation, purification, and characteristics of the sulfurylase/kinase complex from rat chondrosarcoma have been recently described (Lyle et al., 1994a−d). Protein concentrations of all preparations were determined according to the BioRad protein assay protocol.

Generation of Monoclonal Antibodies. Three Balb C mice were injected subcutaneously with 25−30 μg of the most highly purified (Lyle et al., 1994a) enzyme protein in 25 μL of PBS and 135 μL of Freund's adjuvant. After repeated injections at 3 week intervals and a final boost with 80 μg of enzyme protein injected intravenously into the tail vein, the mice were sacrificed and dissociated splenocytes were fused with myeloma (SP2/0) as described by Harlow and Lane (1988). After 17 days, the media from wells with visible colonies were tested by ELISA for immunoreactivity against ATP affinity-purified enzyme preparations. Positive colonies were recultured and retested through several rounds of screening and finally diluted and plated in order to obtain a single cell/well. One strong positive clone, designated 3D5, was determined to be an IgG1 with κ light chain and was used for further studies. Spent media was purified by protein G affinity chromatography or ammonium sulfate precipitation to obtain purified immunoglobulin (Harlow & Lane, 1988).

Western Blot Analysis. Cartilage preparations of both normal and brachymorphic mice were partially purified through an ATP affinity column (Geller et al., 1987). Samples were run on 10% PAGE and transferred to nitrocellulose overnight at 13 mA. The blot was developed according to the chemiluminescence method described by Amersham using the mouse monoclonal antibody against ATP sulfurylase/APS kinase generated in this laboratory.

Enzyme Assays. The sulfurylase assay used was previously described (Lyle et al., 1994c.) The assay reaction proceeds in the physiologically reverse direction of ATP formation. Standard assays contained 50 mM NaH_2PO_4−K_2HPO_4 (pH 7.8), 12 mM $MgCl_2$, 0.5 mM DTT, 5 mM NaF, 0.2 mM $Na_4P_2O_7$ (containing 6.7 μCi of ^{32}P), 0.1 mM APS, and 50 μL of enzyme preparation.

The standard kinase assay was performed as earlier described (Lyle et al., 1994d). A standard assay contained 10 nM [^{35}S]APS (as prepared previously; Geller et al., 1987), 250 μM ATP (pH 7.0), 5 mM $MgCl_2$, 10 mM ammonium sulfate, and 12 μL of enzyme and was brought to 25 μL with buffer A. For comparison with the coupled assay, the reaction mixture contained 10 mM ATP, 20 mM $MgCl_2$, 22 mM Tris-HCl (pH 8.0), 10 μL of enzyme preparation, and [^{35}S]APS at the steady-state level measured for the coupled assay (see Results).

The coupled assay has been recently developed (Lyle et al., 1994a). 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. For isotope dilution experiments, the reaction was started as above, and unlabeled APS was added at either time zero or after 4 min. For isotope enrichment, the reaction was started as above with unlabeled SO_4^{2-}, and [^{35}S]APS was added.

Kinetic Data Analysis. Kinetic data were processed by unweighted iterative, nonlinear least squares fitting of initial velocities as a rectangular hyperbolic function of substrate concentration. The apparent kinetic parameters obtained were then used to construct the initial velocity patterns. Kinetic constants were derived from nonlinear least squares fitting of secondary plots of the best-fit parameters from the primary data pattern using the appropriate rate equations.

RESULTS

Monoclonal Antibody Reactivity to Mouse Sulfurylase/Kinase. There is substantial evidence that both ATP sulfurylase and APS kinase activities are reduced in the brachymorphic mutant (Schwartz et al., 1978; Sugahara & Schwartz, 1979, 1982). In order to better understand the nature of this double enzyme defect, immunologic reagents were prepared to probe and purify the activities as well as to screen expression libraries in order to clone the genes coding for these enzymes.

These antibodies were recently used in immunoaffinity chromatography and Western blot studies to show that ATP sulfurylase and APS kinase from rat chondrosarcoma reside on a single, bifunctional protein of 56 kDa (Lyle et al., 1994a). These antibody reagents show strong cross-reactivity with the same activities from mouse cartilage. Using both polyclonal and monoclonal antibody reagents, we first tested whether less enzyme protein is synthesized, or whether a defective protein exhibiting lower activity is produced by the mutant mouse. When enzyme preparations containing equivalent amounts of APS kinase activity were run on 10% PAGE and Western blotted, the monoclonal antibody recognized a single protein band at 56 kDa (Figure 1). These results suggest that ATP sulfurylase and APS kinase in both the normal and brachymorphic mouse do not significantly differ in size or overall properties. Furthermore, the band from the brachymorphic preparation exhibited significantly more immunoreactivity than the corresponding band in the normal preparation. Similar results were obtained when equal amounts of normal and mutant ATP sulfurylase activity were subjected to the same procedures. These findings indicate that more enzyme protein from the brachymorphic preparation is needed to achieve the same level of activity as the enzyme from normal tissue. In contrast, when comparable amounts of protein from a normal and a brachymorphic preparation (which contained significantly lower levels of kinase activity) were loaded, the immunoreactivity detected was equivalent in the two preparations (data not shown). Thus, it appears that the brachymorphic defect results in the production of the normal amount of a dysfunctional enzyme.

Kinetic Behavior of Sulfate Activation Pathway. In order to begin to understand how this mutation affects both ATP

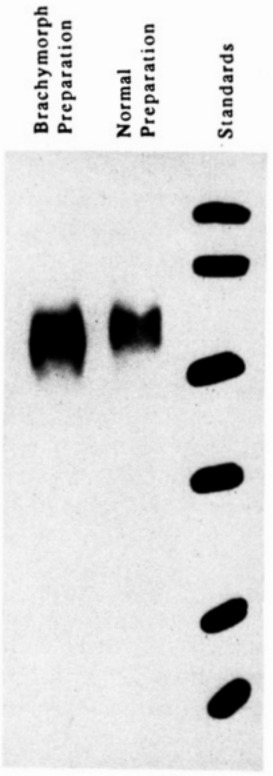

FIGURE 1: Comparison of ATP sulfurylase and APS kinase from normal and brachymorphic mouse cartilage by Western blot. Direct comparison of the ATP affinity column-purified preparations of the normal (C57B1/6J) and brachymorphic mouse. Equal amounts of APS kinase activity were loaded in each lane. The standards, visualized by staining this lane with amido black, are in the low molecular mass range: 97 400 Da; 66 200 Da; 45 000 Da; 31 000 Da; 21 500 Da; 14 400 Da.

sulfurylase and APS kinase activities of the brachymorphic mouse, a mechanistic analysis of the individual reactions was undertaken. Prior to analysis, activity levels in mutant cartilage were determined to confirm the previously observed deficiency in these samples, *i.e.*, ATP sulfurylase was approximately 50% of normal activity. Because of thermodynamic constraints, the kinetic assay reaction was performed in the physiologically reverse direction, toward the production of ATP and SO_4^{2-} from APS and PP_i. APS and PP_i were varied with respect to each other to obtain initial velocity patterns. Since it has been previously determined that the individual reaction mechanism for ATP sulfurylase from rat chondrosarcoma is sequential, ordered with APS as the first substrate to bind in the reverse direction (Lyle et al., 1994c), the initial velocity data were modeled according to the rate equation:

$$1/V_0 = K_m^{APS}/(V_{max}[APS]) + K_m^{PP_i}/(V_{max}[PP_i]) + K_x/(V_{max}[PP_i][APS]) + 1/V_{max} \quad (1)$$

where $K_x = K_s^{APS}K_m^{PP_i}$. Values corresponding to the slopes and intercepts of the double reciprocal plots were computed using the nonlinear least squares program and were subsequently graphed by the same statistical procedures in secondary plots. The kinetic coefficients generated from these data are given in Table 1. Due to APS substrate inhibition at high concentrations in this system, the levels of APS needed to accurately determine intercepts of the secondary plots of the normal enzyme were unapproachable, and thus the intercepts were indistinguishable from the origin.

Table 1: Kinetic Coefficients of ATP Sulfurylase

constant[a]	normal[b]	bm/bm[b]
K_m^{APS}		19.2 ± 4.8
$K_m^{PP_i}$		84.8 ± 21.7
K_x ($=K_s^{APS}K_m^{PP_i}$)		181 ± 46
V_{max}		136 ± 36
V_{max}/K_m^{APS}	4.39 ± 0.2	7.09 ± 0.01
V_{max}/K_x	0.30 ± 0.01	0.75 ± 0.32

[a] K_m values are given in μM, K_x in μM^2, V_{max} in nmol/(min·g of protein), V_{max}/K_m^{APS} in nmol/(μM·min·g of protein), and V_{max}/K_x in nmol/(μM^2·min·g of protein). [b] Values were determined by modeling computer-generated lines to the data set using the rate equation described in Results.

Table 2: Kinetic Coefficients of APS Kinase

constant[a]	normal	bm/bm
K_m^{APS}	0.41 ± 0.04	0.140 ± 0.03
K_m^{ATP}	40 ± 3	25 ± 2
K_x ($=K_s^{APS}K_m^{ATP}$)	9.8 ± 2.3	7.4 ± 0.9
V_{max}	44.6 ± 0.7	3.5 ± 0.2

[a] K_m values are given in μM, K_x in μM^2, and V_{max} in nmol/(min·g of protein).

Therefore, the V_{max} term was not defined, and the K_m values for the normal cartilage were not obtainable. However, the slopes of the secondary plots provide the ratios V_{max}/K_m^{APS} and V_{max}/K_x for comparison with the ratios for the mutant cartilage (Table 1).

The mutant kinase activity (14% of normal) was analyzed in a similar manner. The mechanism of the individual APS kinase reaction of rat chondrosarcoma has also been shown to be sequential, ordered (Lyle et al., 1994d). Thus, the kinetic coefficients of mouse APS kinase were evaluated according to the equation:

$$1/V_0 = K_m^{APS}/(V_{max}[APS]) + K_m^{ATP}/(V_{max}[ATP]) + K_x/(V_{max}[ATP][APS]) + 1/V_{max} \quad (2)$$

where $K_x = K_s^{APS}K_m^{ATP}$. The kinetic coefficients for the kinases from normal and brachymorphic cartilage were compared, and Table 2 shows that both the V_{max} and K_m^{APS} for the mutant are lower than for the normal.

To further investigate the nature of the defect, the production of [^{35}S]APS and [^{35}S]PAPS over time was followed by incubating free [^{35}S]SO_4^{2-} and ATP with enzyme preparations containing both ATP sulfurylase and APS kinase and stopping the reaction at fixed time points (Figure 2). The mutant cartilage showed a decrease of 50−60% in the initial velocity of APS production, but the steady-state concentration of APS reached a maximum level of 300−400 nM for both mutant and normal cartilage. In the mutant, PAPS production over time was reduced to approximately 20% of the normal amount.

Channeling Defect Shown by Isotope Dilution and Enrichment. To investigate the possibility of intermediate channeling, isotope dilution and enrichment experiments were performed. Unlabeled APS was included with the ATP and labeled sulfate, and the reaction was initiated by adding enzyme; thus, the starting concentration of labeled APS was zero. The pattern of labeled PAPS production in normal and bm/bm preparations is illustrated in Figure 3. For the normal enzymes, the point at which 50% of the PAPS is labeled corresponds to approximately 1.5 μM unlabeled APS

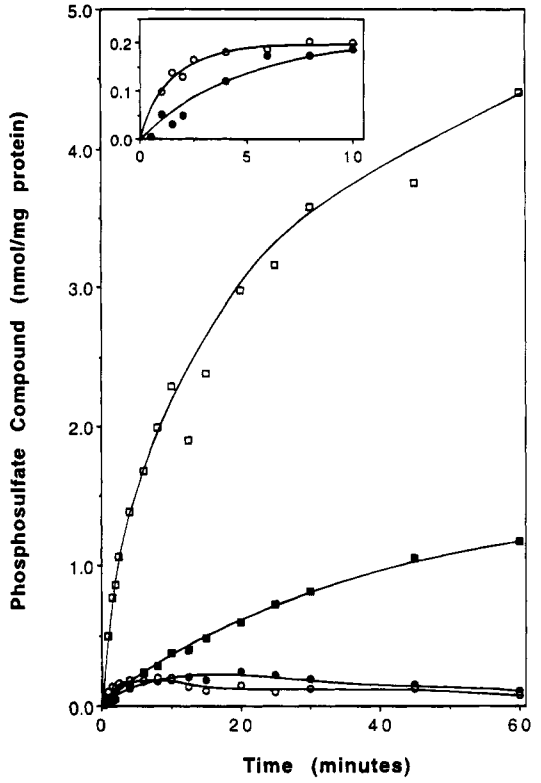

FIGURE 2: Time course of overall assay of ATP sulfurylase and APS kinase of normal and brachymorphic mouse cartilage. Incorporation of $^{35}SO_4^{2-}$ into PAPS, normal (□) or mutant (■), and APS, normal (○) or mutant (●), was measured over time and simultaneously determined by paper electrophoresis. The assays contained 34 μg of protein, 0.4 mM $^{35}SO_4^{2-}$, 10 mM ATP, and 20 mM $MgCl_2$. *Inset*: A blowup of the initial APS formation of normal (○) and mutant (●) preparations.

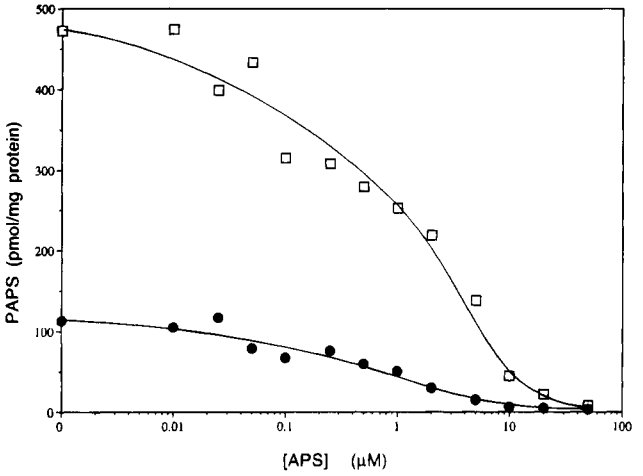

FIGURE 3: Isotope dilution of ATP sulfurylase and APS kinase from normal and brachymorphic mouse cartilage. The production of [^{35}S]PAPS by the sulfate-activation assay of normal (□) and mutant (●) samples was measured at various concentrations of unlabeled APS (prepared fresh in 50 mM Tris-HCl, pH 8.0). The assay contained 0.5 mM $^{35}SO_4^{2-}$, 10 mM ATP, 20 mM $MgCl_2$, and 43.8 μg of normal protein or 47.8 μg of mutant protein. The reaction time was 10 min.

while the steady-state concentration of labeled APS was measured at 79 nM. For the mutant enzymes, the concentration at which 50% of the PAPS is labeled corresponds to only 350 nM unlabeled APS while the steady-state concentration of labeled APS was determined to be 63 nM. Thus, the concentration of exogenous, unlabeled APS which results in 50% dilution is 19-fold higher than the steady-state level of sulfurylase-produced APS for the normal activities but only 5.6-fold higher for the mutant.

Table 3: Isotope Dilution[a]

	[APS] added	pmol of PAPS	% dilution
normal mouse	0	11.8	
	100 nM	11.1	5.9
	500 nM	10.5	11
	5 μM	10.3	13
mutant mouse	0	4.13	
	100 nM	3.66	11
	500 nM	3.19	23
	5 μM	1.81	56

[a] Observed reduction in the amount of labeled PAPS produced when unlabeled APS was added to the reaction mixture (10 mM ATP, 0.4 mM [^{35}S]SO_4, 20 mM $MgCl_2$, and 25.4 μg of mutant protein or 24 μg of normal protein) at 4 min and the reaction was stopped at 10 min. Steady-state levels of APS were 120 and 216 nM for normal and mutant assays, respectively.

Although the steady-state level of APS appears to be constant from as early as 2 min, the starting concentration is zero. Thus, the ratio of exogenous to sulfurylase-produced 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 APS had been achieved. A range of concentrations of unlabeled APS was added to the sulfate-activation assay initiated with ATP and labeled sulfate, and representative results are displayed in Table 3. Isotope dilution of the normal enzyme by unlabeled exogenous APS reached only 13% at a concentration of 5 μM. For the brachymorphic tissue, the isotope dilution was 56% with a concentration of 5 μM unlabeled APS. As before, much less exogenous APS is needed to disrupt the channeling mechanism in the mutant system.

Isotope enrichment experiments produced similar results. When the ratios of exogenous [^{35}S]APS to endogenously-produced APS were comparable (42% for both normal and mutant), there was nearly a 2-fold increase in the amount of labeled PAPS produced by the mutant system (2.4% in normal vs 4.1% in mutant). This difference in enrichment also suggests that exogenous APS has a greater access to the kinase active site in the brachymorphic enzyme, resulting in a decrease in the channeling efficiency of the PAPS synthesis system in this mutant.

Channeling between the enzymes in this pathway was further examined by comparing the initial velocity of PAPS production in the APS kinase reaction alone to the velocity in the combined system. In the coupled assay, the kinase activity utilizes APS produced from ATP and [^{35}S]SO_4 by the sulfurylase enzyme, while in the isolated kinase assay APS is added exogenously to the reaction mixture. For normal cartilage, the initial velocity of PAPS production was 45-fold greater in the combined system than in the kinase reaction alone. This indicates a greater efficiency in utilization of APS in the overall system due to the channeling of the intermediate between ATP sulfurylase and APS kinase. In the brachymorphic system, the initial velocity for the overall reaction was only 25-fold greater than that in the kinase reaction alone. A measure of the efficiency of channeling can also be obtained by comparing the initial rate of PAPS production to the initial rate of APS production

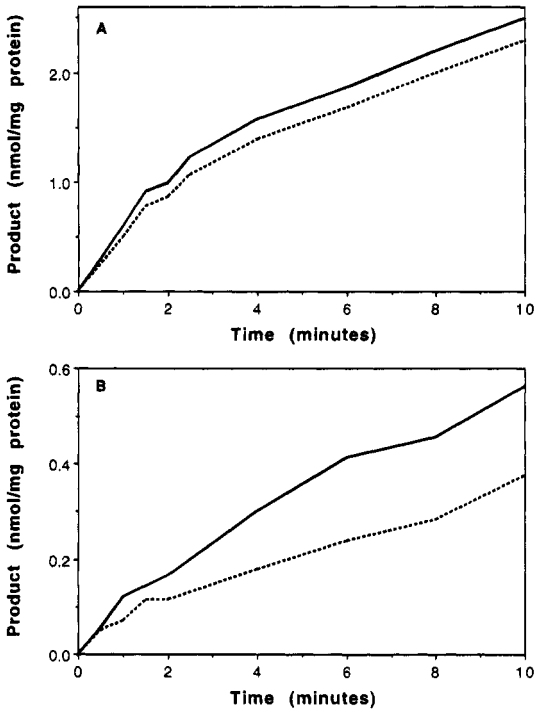

FIGURE 4: Appearance of products in the overall assay of ATP sulfurylase and APS kinase from normal and brachymorphic mouse cartilage. The total APS produced, APS + PAPS measured (solid line), is compared to the amount of PAPS alone (dashed line). (A) Normal, (B) mutant. The assays contained 34 μg of protein, 0.4 mM $^{35}SO_4^{2-}$, 10 mM ATP, and 20 mM $MgCl_2$.

(APS + PAPS measured) in the combined system. Calculated in this way, the channeling efficiency of normal cartilage is approximately 90% while that of mutant cartilage is only 54% (Figure 4).

DISCUSSION

It is intriguing that a presumed single mutation affects the activities of both ATP sulfurylase and APS kinase in the brachymorphic mutant. Geller et al. (1987) found these activities to copurify, to have nearly identical behavior, and to share the common substrates, ATP and APS. This suggested that the two activities may be closely associated spatially through enzyme–enzyme interactions, may comprise a multifunctional complex, or may actually reside on a single bifunctional polypeptide. Recent results in this laboratory show that the reactions in rat chondrosarcoma are catalyzed by a single, bifunctional protein (Lyle et al., 1994b). Using newly prepared antibodies which bind both activities, Western blot analysis allowed for the first time a direct comparison of the enzyme complex size and relative amounts of activity in the brachymorphic and normal mouse. Both brachymorphic and normal enzyme preparations, containing ATP sulfurylase and APS kinase activity, exhibit a single immunoreactive band at 56 kDa, which coincides with the single, bifunctional protein obtained from rat chondrosarcoma (Lyle et al., 1994b). Furthermore, when normalized for APS kinase activity, the bm band detected with the antibody probe exhibits a greater amount of immunoreactivity (based on densitometry scanning). Thus, more brachymorphic protein is needed to achieve a level of enzymic activity similar to that of the normal protein, suggesting that a normal amount of functionally defective enzyme, containing both sulfurylase and kinase activities, is produced by the mutant.

The functional significance of the close coupling of the two active sites in a single polypeptide has also been explored. It is well established that since the ATP sulfurylase reaction has an extremely low K_{eq} in the physiologically forward direction (Robbins & Lipmann, 1958a,b), the reaction is thermodynamically favored in the reverse direction. Thus, the APS kinase reaction is needed to remove the APS product of the sulfurylase reaction, thereby lowering the ΔG and promoting the forward reaction. Previous studies have shown that ATP sulfurylase has a high affinity for the APS intermediate (Shoyab & Marx, 1971; Robbins & Lipmann, 1958a,b), consistent with the present finding of a K_m^{APS} in the low micromolar range. In addition, although it had never been previously demonstrated, it had been suggested that APS may not be released free into solution but may remain bound to the enzyme *in vivo* (Shoyab & Marx, 1971; Seubert et al., 1983). Our recent findings show that the two activities are kinetically coupled through a channeling mechanism (Lyle et al., 1994a). Thus the phenomenon of channeling, which allows the product of a first reaction to remain in the immediate vicinity of the enzyme while becoming the substrate for a subsequent reaction, was considered as a possible functional location for the manifestation of the brachymorphic mutation.

In order to explore this possibility, a detailed kinetic analysis of each individual reaction as well as the overall pathway of sulfate activation was undertaken, since differences between the normal and mutant enzymes should be revealed by comparing the enzymatic behavior. The initial velocity patterns of bisubstrate variation experiments indicate a sequential reaction for normal and bm cartilage ATP sulfurylase and agree with earlier studies of the rat chondrosarcoma enzyme (Lyle et al., 1994c), where ATP sulfurylase follows a ternary-complex, ordered mechanism with APS as the leading substrate for the reaction in the physiologically reverse direction. Although the V_{max} term was not obtainable for the enzyme from normal mouse cartilage, an estimate of the apparent $K_m^{PP_i}$ values at subsaturating APS concentrations can be obtained from the initial velocity plots (data not shown). It is evident from such a comparison that $K_m^{PP_i}$ values for the normal and mutant enzymes are unlikely to differ. In addition, the ratios V_{max}/K_x and V_{max}/K_m^{APS} were compared to the same ratios calculated from the determined constants for the mutant enzyme and show that the values of the mutant are higher than those for the normal. Therefore, it is likely that the K_m^{APS} and K_x for the mutant are lower than those for the normal.

The initial velocity patterns for APS kinase show that this enzyme also acts by a ternary-complex mechanism, commensurate with our earlier work on APS kinase from rat chondrosarcoma (Lyle et al., 1994d). The constants presented in Table 2 are in the same range as those published for this enzyme from other organisms (Lyle et al., 1994; Renosto et al., 1984; Jender & Schwenn, 1984). As shown, the K_m^{APS} for the mutant kinase is lower than the normal, and the difference is significant at a 95% confidence level. In addition, the V_{max} of the mutant enzyme is significantly lower than normal. Thus, it appears that the mutant sulfurylase is somewhat affected, but the mutant kinase has a markedly lower catalytic activity and also lower K_m value for free APS than the normal enzyme. These results can be further explained in the context of the channeled overall sulfate-activation pathway in mutant and normal cartilage.

The APS used in the kinetic studies of the individual reactions is exogenously added APS. As shown by the channeling data, free APS has a greater ability to enter the pathway in the mutant. The leaky channel allows APS easier access to the activities when measured individually and results in lowered K_m^{APS} values for the mutant activities. The normal activities cannot use free APS as well and therefore have higher K_m values for free APS. Thus, the data from the individual reactions support a defect in the channeling ability of the brachymorphic enzymes.

Isotope dilution experiments 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 APS kinase. If it is assumed that the steady-state level of sulfurylase-produced APS is constant throughout the overall reaction, the concentration of exogenous, unlabeled APS needed to give 50% dilution is approximately 19-fold higher than the level of APS produced by the sulfurylase enzyme in the normal preparation. Thus, if the endogenous and exogenous APS concentrations are equal, one exogenous APS molecule would enter the pathway for every 19 molecules of endogenous APS molecules passing through the system. For the mutant enzyme preparation, the APS concentration which produces 50% dilution is only 5.6-fold higher than the endogenous APS concentration, or one exogenous APS molecule enters for every 5.6 endogenously produced APS molecules. Since the initial concentration of labeled APS is zero and thus the ratio of exogenous to endogenous APS at the determined 50% dilution point is initially much higher, further experiments were performed in which the APS was added after 4 min of the reaction. These isotope dilution and enrichment experiments also clearly exemplify the difference in channeling ability between normal and mutant systems.

Moreover, the other types of experiments used to describe intermediate channeling are consistent with the isotope dilution data and also indicate that the brachymorphic defect causes a decrease in the ability of the enzymes to channel APS and efficiently produce PAPS. Taken together, all of the results indicate that APS produced by the mutant sulfurylase dissociates from the enzyme and more readily mixes with the exogenously added APS before becoming the substrate for the kinase activity. In contrast, APS generated by the normal mouse sulfurylase is efficiently channeled between the active sites, as previously observed in the rat chondrosarcoma system (Lyle et al., 1994a). An alternative possibility that might explain the decrease in channeling and greater dissociation of APS from the enzyme surface could be the significant reduction in V_{max} of the kinase reaction. However, this would not account for the decrease in reverse sulfurylase activity observed in the mutant. Therefore, the defect most likely lies in the channeling function which kinetically links the two activities.

It is intriguing to speculate how a single gene mutation affects the structure of two enzyme activities. Since all current data suggest that the two reactions are catalyzed by a single, bifunctional enzyme, the mutation may affect tertiary structure, causing the activities to be more labile, as in the enzymes orotate phosphoribosyltransferase and OMP deacarboxylase, responsible for the human defect of orotic aciduria (Perry & Jones, 1989). This suggestion is commensurate with the increased cold lability of the brachymorphic kinase (Sugahara & Schwartz, 1982). However, Western blots of normal and brachymorphic cartilage detected no increased breakdown products in the mutant. It is also possible that the two activities share a single APS binding pocket, and the defect may alter this site. Alternatively, the defect may be at a site removed from the substrate binding site, but necessary for a conformational change in the active site during catalytic turnover. Either of these models would explain the decrease in channeling efficiency observed in the brachymorphic enzyme system. Whichever model is eventually established for the PAPS pathway, all the results are consistent with a channeling mechanism for APS as the likely site of the mutation in brachymorphic mice. Current work is focused on determining the physical nature of the functional channel and possible alterations in the brachymorphic system.

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