Journal of the Korean Chemical Society
1998, *Vol. 42, No. 6*
Printed in the Republic of Korea

단 신

쥐 뇌의 ATP황산화 효소의 부분정제와 특성

朴廷培 · 崔明彦*
서울대학교 자연과학대학 화학과
분자촉매 연구센터
(1998. 7. 6 접수)

Partial Purification and Characterization of ATP Sulfurylase in Rat Brain

Jeong-Bae Park and Myung-Un Choi*
Department of Chemistry and Center for Molecular Catalysis, Seoul National University, Seoul 151-742, Korea
(Received July 6, 1998)

Sulfate groups found in a large variety of compounds have been known to exert important biological roles in living organism. Especially in brain, the reactions of sulfation involve in the metabolism of monoamines, the posttranslational modification of tyrosyl protein[1] and neuropeptide cholecystokinin.[2] All these reactions are catalyzed by sulfotransferases which transfer sulfate group from universal sulfate donor of 3'-phosphoadenosine 5'-phosphosulfate (PAPS) to various acceptors. The synthesis of PAPS *in vivo* is carried out by two sulfate-activating enzymes, ATP sulfurylase and adenosine 5'-phosphosulfate (APS) kinase.[3] Because the equilibrium of ATP sulfurylase reaction lies far to the left, the sulfurylase determines the concentration of PAPS *in vivo*.[4]

ATP sulfurylase: $ATP+SO_4^{2-} \rightleftharpoons APS+PPi$, $K_{eq}=10^{-7}$

ATP kinase: $APS+ATP \rightleftharpoons PAPS+ADP$, $K_{eq}=10^{3}$

Since the recognition of existence and role of PAPS, ATP sulfurylase has been characterized in many organisms including yeast,[5] rat liver,[6,7] rat chondrosarcoma,[8,9] *Escherichia coli* K12,[10] spinach,[11] and *Chlamydomonas reinbardtii*.[12] In the study of ATP sulfurylase in *Penicillium chrysogenum*,[13] Segel group clarified some basic questions about the enzyme. It has a regulatory sulfhydryl group[14] and is allosterically inhibited by PAPS.[15] ATP sulfurylase : APS complex does not serve as a substrate for APS kinase.[13] It means that there is no substrate channeling of APS between the two sulfate-activating enzymes in *Penicillium chrysogenum*. However in mammalian source of ATP sulfurylase such as rat chondrosarcoma, the substrate channeling of APS was reported.[9] Recently cDNA encoding ATP sulfurylase-APS kinase has been isolated.[16] In the study of *Escherichia coli* K-12, Liu *et al.* (1998) found that ATP sulfurylase has GTP binding site and that the GTP stimulates ATP sulfurylase activity.[17]

As a part of our effort for studying protein sulfation,[18-21] it was necessary to examine the regulation of PAPS in rat brain tissue. Therefore we attempted to purify and characterize ATP sulfurylase in rat brain.

EXPERIMENTAL

Materials. Rats (Wistar, 4 week-old female) were supplied from Animal Bleeding Laboratory

of Seoul National University. [^{35}S] Sulfate (1,050 Ci/mmol)(sodium salt) was purchased from Amersham and [^{32}P] pyrophosphate (3,000 Ci/mmol) was from New England Nuclear. ATP, PAPS, APS, GTP, phenylalanine, Sephacryl S-300, and alumina C_γ were obtained from Sigma. β-Naphtol was purchased from Yakuri and hydroxylapatite was obtained from Bio-Rad. TSK phenyl-5PW, TSK DEAE-5PW, and TSK G 3000SW columns were purchased from LKB.

Assay of ATP Sulfurylase. The APS dependent conversion of ^{32}PPi to [^{32}P] ATP is the basis of reverse ATP sulfurylase assay.[22] The reaction mixture (100 μL of total volume) contained 50 mM phosphate buffer (pH 6.8), 0.5 mM $MgCl_2$, 10 μM $Na_4P_2O_7$, 5 μM APS, 5 μCi $Na_4[^{32}P]P_2O_7$, and 50 μL of enzyme solution. After 30 min incubation at 37 °C, the reaction was terminated by adding 0.6 mL of ice cold Norit solution (0.33% acid-washed Norit charcoal in 50 mM imidazole buffer (pH 7.0) with 10 mM $Na_4P_2O_7$). After the tubes were centrifuged for 5 min in a table-top centrifuge, the supernatants were discarded and each pellet was washed three times with 0.6 mL of buffer (50 mM imidazole (pH 7.0) with 10 mM $Na_4P_2O_7$).

The final pellets were then dissolved in 0.2 mL of 50% (v/v) ethanol containing 0.1% (v/v) Tween 80 and 0.4% (w/v) NH_4OH. An aliquat of 0.1 mL of the radioactivity of supernatant was determined in a LKB liquid scintillation counter. This assay was used routinely for chromatographic work. For quantitative determination of the enzyme activity ATP formed was monitored with HPLC DEAE chromatography. In this case, the reaction mixture (200 μL of total volume) contained of 50 mM phosphate buffer (pH 6.8), 10 mM $MgCl_2$, 2.5 mM $Na_4P_2O_7$, 0.5 mM APS, and 100 μL of enzyme solution. The reaction was terminated by injecting 50 μL of reaction product into HPLC TSK DEAE-5PW column (8×75 mm). The column was preequilibrated with 10 mM piperazine buffer (pH 5.5) and elution was carried out with a linear gradient of 0-500 mM NaCl at a flow rate of 0.5 mL/min. One unit of enzyme activity was defined as the amount of enzyme that produced 4.2×10^{-10} mol ATP per min.

Determination of Incorporation of $[^{35}S]SO_4^{2-}$. In order to characterize the ATP sulfurylase activity in its natural sulfate substrate the incorporation of $[^{35}S]SO_4^{2-}$ into APS and PAPS was separated by HPLC-DEAE chromatography.[23] The reaction mixture (200 μML of total volume) contained 50 mM phosphate buffer (pH 6.8), 5 mM $MgCl_2$, 5 mM ATP, 0.1 mM Na_2SO_4, 20 μCi $[^{35}S]SO_4^{2-}$ and 50 μL of enzyme solution. After 30 min incubation at 37 °C, the reaction was terminated by injecting 50 μL of reaction product into HPLC TSK DEAE-5PW column. The eluent was collected at a flow rate of 0.5 mL/min and its radioactivity was determined in a LKB liquid scintillation counter.

Purification of ATP Sulfurylase. Wistar rats were sacrificed by decapitation and brains were removed quickly. Eight brains (12 g) were homogenized in 3 volumes of buffer (contained 0.25 M sucrose, 0.08 M $KHCO_3$ and 1 mM mercaptoethanol). The homogenate was centrifuged 100,000× g for 60 min and the supernatant was used as the enzyme source. To this supernatant was added C_γ alumina gel, 10 mg per ml supernatant and stirred for 30 min. After centrifugating for 15 min at 10,000×g, the precipitate was resuspended in the same volume of 0.8 M $(NH_4)_2SO_4$ as the original supernatant and stirred again for 30 min. After the gel was removed by centrifugation for 15 min at 10,000×g, the supernatant was precipitated by adjusting solid ammonium sulfate to 2.9 M. The collected ammonium sulfate precipitate was dissolved in 3 ml of phosphate buffer solution (50 mM phosphate, pH 6.8, with 10 mM mercaptoethanol) and poured to a Sephacryl S-300 column (2×66 cm) which was preequilibrated with the phosphate buffer solution. Fractions of 3 ml per 30 min were collected and the pooled active fractions poured directly to hydroxylapatite column (1×8 cm) which is preequilibrated with the buffer solution with 10% (v/v) glycerol. After washing with 5 bed volume of the preequilibrated buffer, a linear gradient (20 ml each) was developed from 50 mM to 400 mM phosphate containing 10% (v/v) glycerol. Active

fractions of 1 ml per 20 min were pooled and solid ammonium sulfate was added to make 1 M solution. After injection to HPLC TSK phenyl-5PW column (8×75 mm) which is preequilibrated with 1 M phosphate buffer (pH 6.8), 1 M to 0 M phosphate buffer gradient was developed for 60 min in fractions of 1.5 ml per 3 min.

Other Methods. Protein concentration was measured by the method of Bradford using bovine serum albumin as a standard.[24] To determine molecular weight of ATP sulfurylase, HPLC TSK G3000SW column was used. As molecular weight standard, blue dextran (2000 kDa), β-amylase (200 kDa), alcohol dehydrogenase (150 kDa) and bovine serum albumin (66 kDa) were employed.

RESULTS AND DISCUSSION

The first step of purification of cytosolic ATP sulfurylase was carried out with alumina C_γ. Because of high adsorption capacity of alumina C_γ, the specific activity increased up to approximately 5-fold. The alumina C_γ has been used effectively to other sulfate related enzymes.[25] The alumina treated enzyme could be concentrated by precipitation with ammonium sulfate and stored at −70 °C without loss of the enzyme activity. The Sephcryl S-300 gel filtration eliminated most of high molecular weight proteins and ammonium sulfate and the specific activity increased further about 2-fold (*Fig.* 1A). In hydroxylapatite chromatography, ATP sulfurylase activity was eluted between 170 mM and 200 mM of phosphate and the specific activity increased by about 4-fold (*Fig.* 1B). The enzyme activity after the column elution was relatively stable in eluent state with a half life of about one week. As a final step HPLC phenyl chromatography was carried out with TSK phenyl-5PW column placed in an ice bath. The activity was eluted between 600 mM and 450 mM of phosphate (*Fig.* 1C) and the specific activity was 15,000 units/mg protein. From 100,000×g supernatant to the HPLC phenyl chromatography, the purification fold was attained almost 1,900 times. However, this eluent still contained minor

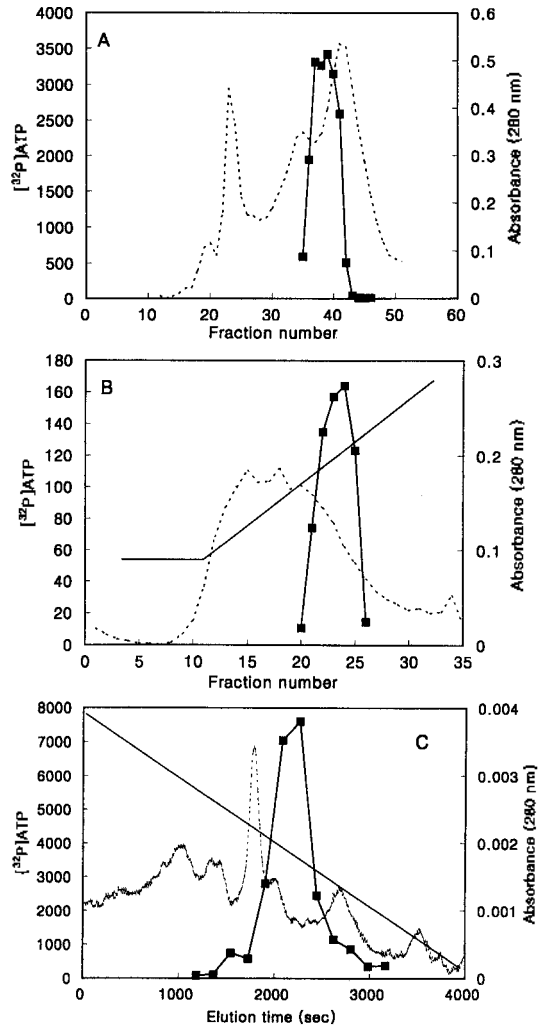

Fig. 1. Purification of ATP sulfurylase. [^{32}P] ATP produced (■) from APS in the presence of ^{32}PPi was measured as the reverse assay of ATP sulfurylase. A, Sephacryl S-300 chromatography; B, Hydroxyapatite chromatography, the gradient of phosphate was carried out from 50 mM to 280 mM; C, HPLC phenyl chromatography with TSK phenyl-5PW, the gradient of phosphate was carried out from 1 M to 50 mM.

impurity, therefore, SDS-PAGE did not show single band (data not shown). The brain ATP sulfurylase in rat brain was stable at phosphate buffer containing 10% glycerol and 1 mM mercaptoethanol, but the activity was rapidly dissapeared at low protein concentration. *Table* 1 summarizes the result of partial purification of

Table 1. Partial purification of ATP sulfurylase in rat brain

	Protein (mg)	Activity (unit)	Specific activity (unit/mg)	Fold
100,000×g supernatant	720	5700	7.92	1
Alumina C_γ and $(NH_4)_2SO_4$ precipitation	81	3200	39.5	5
Sephacryl S-300	22	1900	86.4	11
Hydroxyapatite	1.9	580	305	38
HPLC phenyl	0.005	79	15000	1900

ATP sulfurylase in rat brain.

Molecular weight of ATP sulfurylase in rat brain was about 70 kDa by estimating with TSK G3000SW column (*Fig.* 2). This data is similar to the 68 kDa of crude rat liver enzyme obtained at 100,000×g supernatant.[26] The pH dependence of the purified enzyme was shown in *Fig.* 3A. This profile showed rather broad pH dependence from pH 6.5 to 8.0. The activity increased linearly

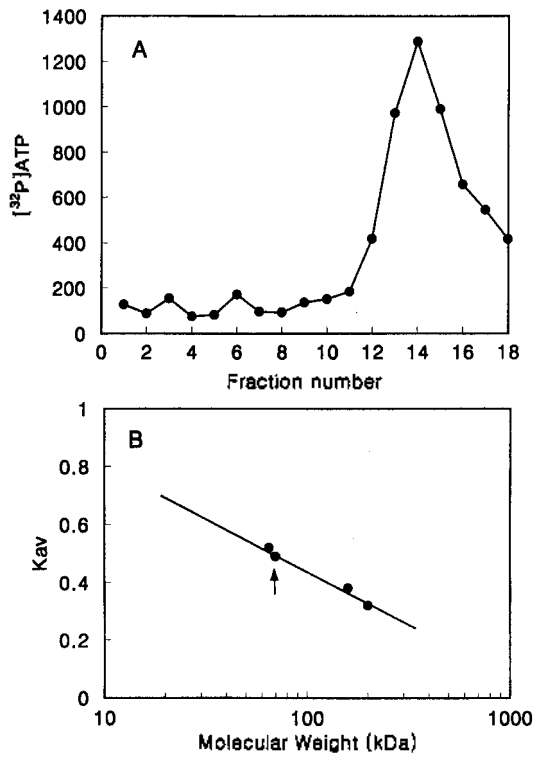

Fig. 2. Molecular weight determination of ATP sulfurylase by HPLC gel filtration chromatography on TSK G3000SW. A, Gel-filtration profile; B, calibration curve for standard proteins, bovine serum albumin (66 kDa), alcohol dehydrogenase (150 kDa), and β-amylase (200 kDa).

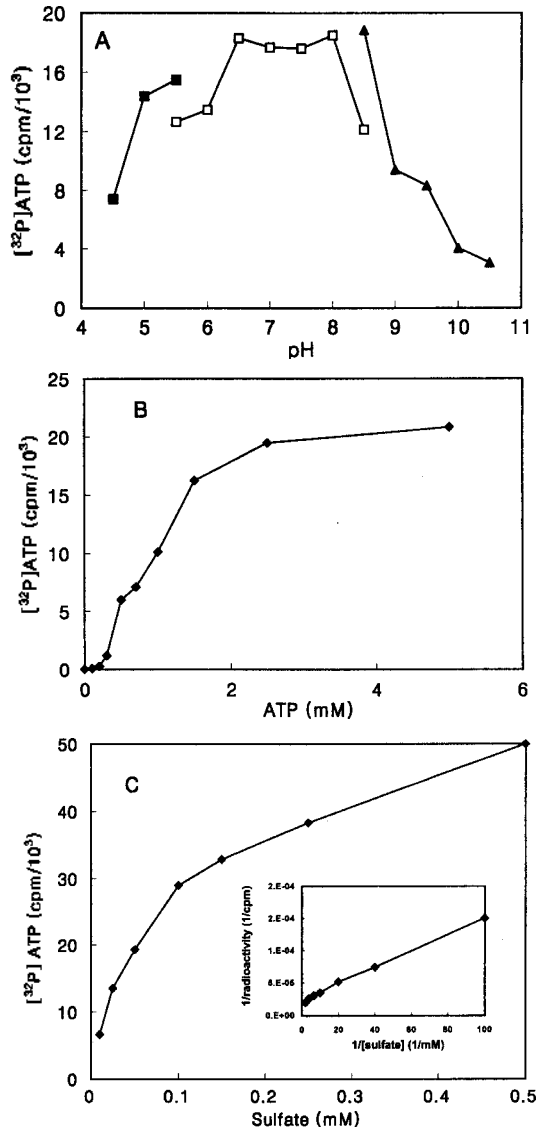

Fig. 3. Some property of ATP sulfurylase in rat brain. A, effect of pH, acetate (■), Tirs-maleate (△), ammonia (▲); B, effect of ATP; C, effect of SO_4^{2-}.

according to incubation time up to 1 hr at 37°C (data not shown). The dependency of ATP on ATP sulfurylase was determined with 0.1 mM SO_4^{2-}. The experimental data revealed a sigmoidal plot (*Fig.* 3B). This result supports a possibility that ATP sulfurylase and APS kinase are coexistent and ATP sulfurylase is allosterically inhibited by PAPS.[15] On the other hand, sulfate dependency with 5 mM ATP showed a typical Michaelis-Menten kinetics (*Fig.* 3C). The Km value for SO_4^{2-} was 92 μM, while Km value reported in the rat liver enzyme was 180 μM.[7] Thus the rat brain enzyme seems to have higher affinity for SO_4^{2-} than liver enzyme. GTP was known to be stimulate ATP sulfurylase of *E. coli* K12,[27] however, no effect was observed in the rat brain. By phenylalnine the 1,900-fold purified ATP sulfurylase was inhibited to 38% of the control activity. This inhibition result is comparable to the 100,000×g supernatant of rat brain which was reported to be 57%.[25] Like other enzymes which use ATP as a substrate, ATP sulfurylase also required Mg^{2+}. Addition of 10 mM EDTA in reaction mixture, the enzyme activity was inhibited more than 90% (data not shown). *Fig.* 4 showed that PAPS was synthesized during the ATP sulfurylase assay. This means the purified enzyme could synthesize simultaneously APS and PAPS. This suggest that the rat brain ATP sulfurylase seemed to be coexistent with APS kinase. This result is similar to that in rat chondrosarcoma, where the two sulfate

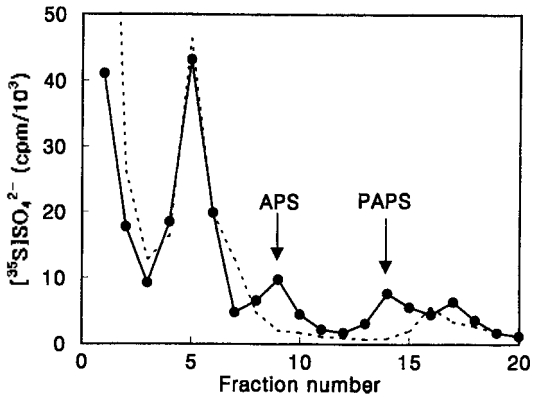

Fig. 4. Separation of reaction products of the ATP sulfurylase by HPLC TSK DEAE-5PW column.

activating enzymes were copurified.[8,9] Recently a cDNA encoding both activities has been isolated from mouse brain.[16]

In summary, the ATP sulfurylase in rat brain was purified 1900-fold in combination of alumina C_f adsorption, ammonium sulfate precipitation, Sephacryl S-300 gel filtration, hydroxylapatite chromatography, and HPLC phenyl chromatography. The molecular weight was estimated to be 70 kDa by gel filtration with HPLC TSK G3000SW. Unlike *E. Coli* enzyme, the activity of brain enzyme was not dependent on GTP, but inhibited by phenylalanine. The capability of brain ATP sulfurylase of synthesizing PAPS implies that the rat brain enzyme also has both sulfate-activating activities like other mammalian sources.

Acknowledgments. This study was supported by grants from the S.N.U. Research Fund and from the Research Institute for Molecular Science. We thank Mr. B. H. Kang for helping the preparation of this manuscript.

REFERENCES

1. Huttner, W. B.; Baeuerle, P. A. *Modern Cell Biology*, **1988**, Vol. 6. Alan R. Liss Inc., New York, pp. 97-140.
2. Vargas, F. *J. Neurochem.* **1990**, *54*, 809.
3. Lipmann, F. *Science* **1958**, *128*, 575.
4. Brion, F.; Schwartz, J.-C.; Vargas, F. *J. Neurochem.* **1987**, *48*, 1171.
5. Robbins, P. W.; Lipmann, F. *J. Biol. Chem.* **1958**, *233*, 686.
6. Burnell, H. N.; Roy, A. B. *Biochim. Biophys. Acta* **1978**, *527*, 239.
7. Yu, M.; Martin, R. L.; Jain, S.; Chen, L. J.; Segel, I. H. *Arch. Biochem. Biophys.* **1989**, *269*, 156.
8. Geller, D. H.; Henry, J. G.; Belch, J.; Schwartz, N. B. *J. Biol. Chem.* **1987**, *262*, 7374.
9. Lyle, S.; Stanczak, J.; Ng, K.; Schwartz, N. B. *Biochemistry* **1994**, *33*, 5920.
10. Leyh, T. S.; Taylor, J. C.; Markham, G. D. *J. Biol. Chem.* **1988**, *263*, 2409.
11. Renosto, F.; Patel, H. C.; Martin, R. L.; Thomassian, C.; Zimmerman, G.; Segel, I. H. *Arch. Biochem. Biophys.* **1993**, *307*, 272.

12. Yildiz, F. H.; Davies, J. P.; Grossman, A. *Plant Physiol.* **1996**, *112*, 669.
13. Renosto, F., Martin, R. L. and Segel, I. H. *J. Biol. Chem.* **1989**, *264*, 9433.
14. Martin, R. L.; Daley, L. A.; Lovric, Z.; Wailes, L. M.; Renosto, F.; Segel, I. H. *J. Biol. Chem.* **1989**, *264*, 11768.
15. Renosto, F.; Martin, R. L.; Wailes, L. M.; Daley, L. A.; Segel, I. H. *J. Biol. Chem.* **1990**, *265*, 10300.
16. Li, H.; Deyrup, A.; Mensch, Jr. J. R.; Domowicz, M.; Konstantiniids, A. K.; Schwartz, N. B. *J. Biol. Chem.* **1995**, *270*, 29453.
17. Liu, C.; Wang, R.; Varlamova, O.; Leyh, T. S. *Biochemistry* **1998**, *37*, 3886.
18. Lee, K.; Choi, M. *Korean Biochem. J.* **1989**, *22*, 91.
19. Ryu, J.; Choi, M. *Korean J. Zool.* **1990**, *33*, 468.
20. Lee, J.; Choi, M. *Korean Biochem. J.* **1990**, *23*, 62.
21. Lee, J. R.; Choi, M. *Korean Biochem. J.* **1992**, *25*, 158.
22. Segel, I. H.; Renosto, F.; Seubert, P. A. *Methods Enzymol.* **1987**, *143*, 334.
23. Hommes, F. A.; Moss, L. *Anal. Biochem.* **1986**, *154*, 100.
24. Bradford, M. M. *Anal. Biochem.* **1976**, *72*, 248.
25. (a) Foldes, A.; Meek, J. L. *Biochim. Biophys. Acta* **1973**, *327*, 365. (b) Hommes, F. A.; Moss, L.; Touchton, J. *Biochim. Biophys. Acta* **1987**, *924*, 270.
26. Matsuo, K.; Moss, L.; Hommes, F. A. *Neurochem. Res.* **1987**, *12*, 345.
27. Leyh, T. S.; Suo, Y. *J. Biol. Chem.* **1992**, *267*, 542.