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ASSAY OF ATP-SULFURYLASE ACTIVITY FROM RAT LIVER BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

Alteration of proteoglycan composition is known to accompany morphogenesis. In many tissues one such alteration is the removal of hyaluronate and its replacement with a sulfated proteoglycan. Several mechanisms that could regulate this alteration have been studied leading to a hypothesis that the increase in the sulfated proteoglycan is regulated by controlling the activity of those enzymes involved in the activation of the sulfate. To measure any variations in these activities usually begins with a purification of the enzyme. However, this procedure is difficult to perform where tissue samples are difficult to obtain in large enough quantities. Therefore, the examination of an enzymatic activity when tissue samples are in short supply requires the development of methods for the assay of the specific activity after a minimum of purification. In this paper we report on the development of just such an assay for ATP-sulfurylase, the enzyme that catalyses the first step in the activation of sulfate. This method uses anion-exchange high-performance liquid chromatography and differs from a previously published procedure [F.A. Hommes and L. Moss, Anal. Biochem., 154 (1986) 100] in that the compounds are detected spectrophotometrically instead of radiometrically and also in that the ATP, ADP, AMP and their sulfated analogues, adenosine 5′-phosphosulfate and 3′-phosphoadenosine 5′-phosphosulfate, are separated isocratically. Studies performed with $^{35}SO_4^{2-}$ were used to validate this new method The separation of all these compounds has allowed us to develop a one-step, on-line assay procedure which can be performed on small samples of partially purified preparations. We have used this procedure to measure the activity of the ATP sulfurylase in extracts of rat liver and tongue. Our results indicated that the ATP-sulfurylase activity from rat liver was soluble with a pH optimum of 8.0. The identity of the reaction product was verified using radiolabeled sulfate as the substrate and recovering the radiolabel in the product. Preliminary kinetic studies with this method showed the sulfurylase activity to have an apparent Michaelis constant of 3 μM and a maximal velocity of 0.56 pmol/min per mg protein.

INTRODUCTION

Proteoglycans are macromolecules present in varying amounts in all connective tissues [1]. The glycosaminoglycan (GAG) side-chains of the proteoglycan

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molecule have been studied extensively with regard to their structure and function [1,2] and have been shown to stimulate cellular aggregation [3], to enhance adhesion [4], to regulate cellular proliferation and differentiation [2,5–7] and to have a role in morphogenesis [2,8,9]. These functions are often associated with qualitative and quantitative modifications of GAG side-chains of proteoglycans [2,8,9].

Studies of chick cornea morphogenesis have indicated that the keratin sulfate, the major GAG of vertebrate cornea synthesized by the corneal fibroblasts in later periods of development, has a higher degree of sulfation than those GAGs made during earlier stages of development [10,11]. Since these biochemicals' modifications also occur during the period of development when the embryonic cornea is becoming transparent, these findings suggested a link between the biochemical events regulating sulfation and morphological processes [9–11]. This conclusion is supported by other studies which showed that cartilage defects in brachymorphic (bm/bm) mice were due to undersulfation of cartilage-specific proteoglycans [12].

The reactions involved in the sulfation of GAGs are shown in Fig. 1. Previous studies have shown that the increase in sulfation of cornea-specific GAG molecules is a result of the availability of 3′-phosphoadenosine 5′-phosphosulfate (PAPS) rather than a change in the specific activity of the GAG sulfotransferase (see Fig. 1), the enzyme that transfers activated sulfate from PAPS to the proteoglycan [9–11]. Consistent with this explanation was the finding that the undersulfation of the proteoglycans in cartilage resulted from a decrease in the level of PAPS rather than a decrease in the activity of the GAG sulfotransferase [13,14]. Because of these results, attention has shifted to those two enzymes required for the activation to sulfate, namely the ATP:sulfate adenylyltransferase (EC 2.7.7.4, ATP-sulfurylase) and ATP:adenylylsulfate phosphotransferase (EC 2.7.1.25, APS-kinase). These reactions are shown in Fig. 1.

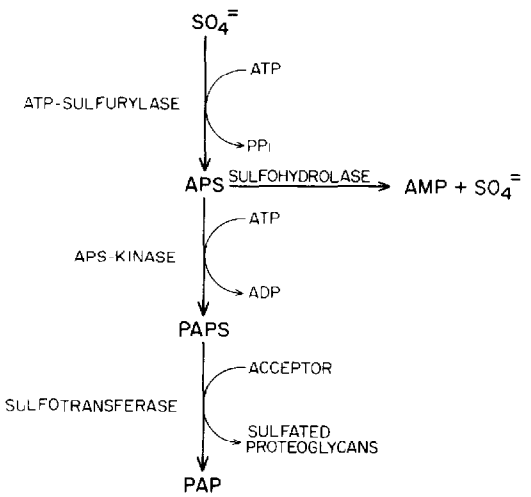

Fig. 1. Pathway involved in sulfate activation and metabolism.

In this paper we have focused on the ATP-sulfurylase reaction. Methods previously developed for the assay of this activity followed, in some cases, the formation of pyrophosphate or, in others, radiolabeled APS [9–15]. However, for these methods to be useful it was necessary to purify the ATP-sulfurylase free of other activities (see Fig. 1 for these reactions). Since our supply of tissue was limited, it was necessary to develop a method that would permit us to measure activity after a minimal amount of purification.

High-performance liquid chromatography (HPLC) is a technique that has been used to assay the activity of many enzymes in crude extracts and in partially purified preparations [16]. The ability to separate completely a number of related components within minutes has made this technique ideal for these assays. A previous study reported the separation of adenosine 5′-phosphosulfate (APS) by anion-exchange HPLC, eluting the column with a step gradient using sodium sulfate as the salt [15]. In contrast, in the present study we have been able to achieve a similar and, with some compounds, an improved separation on anion-exchange HPLC using isocratic elution with sodium bicarbonate as the salt. Using this technique we have been able to separate and quantitate the amounts of ATP, APS, PAPS, ADP and AMP in the reaction mixtures with sufficient reproducibility to perform kinetic studies on the ATP-sulfurylase activity in several tissues from the rat.

EXPERIMENTAL

Materials

ATP, ADP, AMP, APS, PAPS, Trizma base and the enzyme inorganic pyrophosphatase (50 U/ml) were obtained from Sigma (St. Louis, MO, U.S.A.). Adult rats were obtained from Charles River (Wilmington, MA, U.S.A.). $H_2{}^{35}SO_4$ (1 mCi/ml carrier free) was obtained from New England Nuclear (Wilmington, DE, U.S.A.). Sephadex G-25 was from Pharmacia (Piscataway, NJ, U.S.A.).

Instrumentation

The HPLC system consisted of a Model 6000A solvent delivery system from Waters Assoc. (New Milford, MA, U.S.A.), a Model 440 UV absorbance variable-wavelength detector from Waters Assoc. and a Model 7125 injection valve from Rheodyne (Cotati, CA, U.S.A.) with a 50-μl sample loop. Chromatograms were recorded with an Omniscribe dual-pen chart recorder from Houston Instruments (Austin, TX, U.S.A.).

Enzyme preparation

Since preliminary experiments showed that the enzyme for sulfate activation was less active when taken from organs that had been frozen, liver and tongue obtained from a freshly sacrificed animal were used for most of these experiments. After removal, the wet weight of tissues was determined, minced and homogenized using a glass homogenizer at a final concentration of 1 mg/ml in 0.05 *M* Tris–HCl (pH 8), at 4 °C. The homogenate was centrifuged at 30 000 *g* for 10 min and the supernatant fluid removed and centrifuged again for an additional

10 min at 30 000 *g*. Samples of this second supernatant solution were used in the assay for enzymatic activity. Protein content was determined using Coomassie Brilliant Blue stain with bovine serum albumin as the standard [17].

Enzyme assay

The reaction mixture contained in a final volume of 350 μl, 30 μmol Tris–HCl (pH 8), 0.9 μmol ATP, 3 μmol magnesium sulfate, 6 μmol sodium fluoride and 50 μl (2.5 U) of inorganic pyrophosphatase. The reaction was initiated by addition of 50 μl (19 mg protein) of the second supernatant solution and incubated at 37°C. Reactions were terminated by removing samples from the incubation mixture, transferring them to glass test tubes, which were capped and heated to 155°C for 5 min using procedures previously described [16]. Control experiments showed that APS was not degraded at this temperature for 5 min. Precipitated protein was removed from these samples by filtration using Millex-HA filters (0.45 μm pore size). Usually 50 μl of the filtrate were injected for analysis. After separation, the amount of product (APS) was determined from the area of APS peak on the chromatogram.

Ion-exchange HPLC

For anion-exchange HPLC, the chromatographic column was a prepacked Synchropak AX-100 (Synchrom, Lafayette, IN, U.S.A.) (250 mm $\times$4.1 mm I.D.) protected by a guard column packed with Synchrom ASC. The nucleotides were eluted isocratically with a mobile phase containing 0.023 *M* NaH_2PO_4, 0.077 *M* Na_2HPO_4 (pH 7.3) and 0.8 *M* $NaHCO_3$. The flow-rate was 1.0 ml/min and the effluent profile was monitored at 254 nm. When radioactive sulfate was used as the substrate, fractions were collected and the radioactivity determined by scintillation counting.

RESULTS

Initial experiments were performed to establish conditions for the separation of the several adenosine nucleotides that were either substrates or products. A representative chromatogram of the separation of ATP from the products of the primary reaction, namely APS and PAPS, is shown in Fig. 2. Also shown is the separation of these compounds from ADP and AMP, the products of ATPase or sulfohydrolase activities, respectively. These separations were obtained on an AX-100 anion-exchange column using a phosphate-buffered mobile phase containing 0.8 *M* sodium bicarbonate. In other experiments we found that concentrations of sodium bicarbonate more than 0.8 *M* resulted in less separation between ADP and APS, while concentrations less than 0.8 *M* resulted in a prolonged retention time for ATP and PAPS. As expected with ion-exchange HPLC, the retention time was dependent on the number of charges on the nucleotides; thus ADP with two charges was eluted before ATP with three. However, APS was eluted after ADP and PAPS was eluted after ATP indicating that for compounds of similar charge, the charge state of the sulfate increased the retention time.

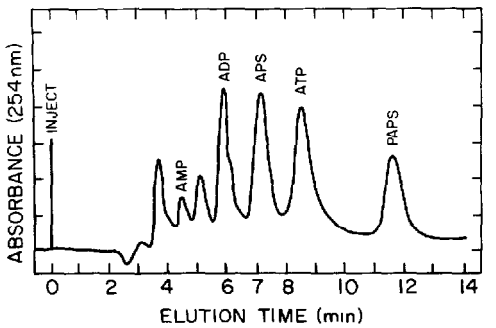

Fig. 2. Chromatogram showing the separation of authentic adenine nucleotides and sulfated derivatives. A solution, containing approximately 100 μM of each compound, was prepared and 400 μl were analyzed. Separation was achieved on an anion-exchange column (Synchropak AX-100) eluted isocratically with a phosphate-buffered mobile phase (pH 7.3) containing 0.8 M sodium bicarbonate. The effluent was monitored at 254 nm.

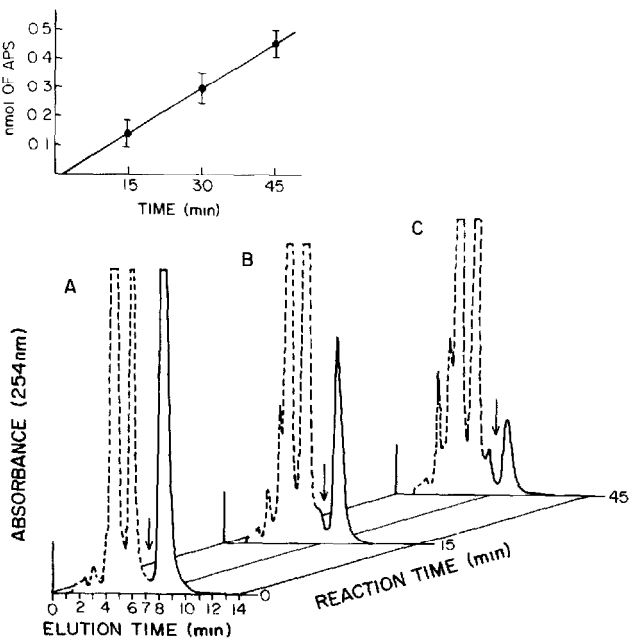

Fig. 3. Time course of formation of APS by activity in rat liver. The reaction mixture contained, in a final volume of 350 μl, 30 μmol Tris-HCl (pH 8.0), 0.9 μmol ATP, 3 μmol magnesium sulfate, 6 μmol sodium fluoride and 50 μl inorganic pyrophosphatase (2.5 U). A 50-μl supernatant sample (19 mg protein) from rat liver was added to start the reaction, then samples were removed at intervals, and the reaction was terminated and analyzed. Chromatograms were obtained after incubation for (A) 0 min, (B) 15 min and (C) 45 min. The arrow indicates the elution time for the reaction product APS.

Initial identification of ATP-sulfurylase in extracts of liver and tongue

The enzymatic activation of sulfate was studied using extracts prepared from rat liver and tongue. The addition of a sample of either extract to a reaction mixture containing ATP and sulfate resulted in the time-dependent formation of

APS as shown by a comparison of the chromatograms in Fig. 3A, B and C. The amount of product formed at each time point was determined from the chromatogram by measuring the area associated with the APS peak (Fig. 3 arrow). These values are plotted in the inset to Fig. 3. Note that the production of APS remains linear even after the ATP level has declined (Fig. 3C). Evidence for the enzymatic activation of the sulfate was derived from other experiments which showed the absence of a product when the extract was either omitted from the reaction mixture or boiled prior to its addition.

Initially, identification of the reaction product as APS was based on the equivalence of the retention time of the reaction product to that of authentic APS as indicated by the arrow in Fig. 3. Further support for the identification of the product was derived from experiments with radiolabeled sulfate (see below).

Initial optimization of the reaction conditions

The chromatograms in Fig. 3 also revealed the time dependence of the formation of other components during the incubation. In our initial experiments the predominant reaction product was ADP, the product of an active ATPase also present in these extracts. As previously reported [9,18,19], we found that the addition of sodium fluoride to the reaction mixture reduced the level of ADP consistent with an inhibition of the ATPase activity. The results of separate experiments showed that, in the presence of sodium fluoride and the absence of sulfate, the production of ADP and AMP from contaminating ATPase was reduced over 90%. We also found that inorganic pyrophosphatase, added to the reaction mixture, increased the amount of APS formed consistent with the interpretation that the reaction was favored by the hydrolysis of pyrophosphate (see Fig. 1). Of course the addition of this activity to our reaction mixture precluded our following the reaction by measuring inorganic orthophosphate (P_i) levels as described by others [14]. In experiments with extracts prepared from tongue, excessive amounts of AMP were formed indicative of the presence of an APS-sulfohydrolase activity. Previous studies showed that this activity in rat liver had a pH maximum of 9.5 [20]. Since we found that the sulfohydrolase activity in tongue was more active than that in extracts from liver, the latter was used for subsequent studies.

The effect of pH (in the range 6–9) and of temperature (in the range 23–40°C) on activity were studied. We found maximum activity at pH 8 and a temperature of 37°C. The series of chromatograms shown in Fig. 3 represents the formation of APS during the course of the incubation using optimized conditions for the activity in rat liver.

Formation of APS from radioactive sulfate

Initially, evidence for the formation of APS was based on the equivalence of the retention time of the reaction product to that of the authentic APS as shown in Fig. 3. To confirm the identity of the reaction product directly, $^{35}SO_4^{2-}$ was used as the substrate with the expectation that the reaction product (APS) would contain radioactive sulfate. In this set of experiments, the reaction mixture contained 6 μCi of $^{35}SO_4^{2-}$ and ATP. The chromatographic profiles, obtained after

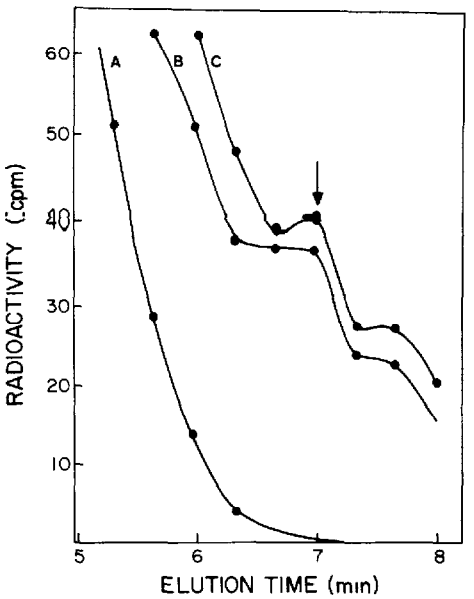

Fig. 4. Formation of [^{35}S]APS. The reaction contained the reagents listed in the legend to Fig. 3 plus 6 μCi $^{35}SO_4^{2-}$. The reaction was started by the addition of extract and after the appropriate interval samples were removed, the reaction was terminated and analyzed by HPLC as described in the legend to Fig. 1. Fractions (300 μl) were collected and 200 μl of each were added to 2 ml of scintillation fluid for analysis of radioactivity. Illustrated are the radioactive profiles of samples removed (A) 0 min, (B) 15 min and (C) 45 min after the start of the reaction. The arrow indicates the expected position of authentic APS.

0, 15 and 45 min of incubation, are shown in Fig. 4A, B and C, respectively. The expected elution position for APS was determined on the basis of the retention time of authentic APS (see arrow in Fig. 4). Fig. 4A shows an analysis of the radiolabeled components present in the reaction mixture at time zero. As expected, a peak of radioactivity representing the unreacted free sulfate is observed but radioactivity was not detected at the APS position at this time. However, as shown in Fig. 4B and C, while unreacted free sulfate continues to be eluted. As the reaction proceeded a radioactive shoulder appeared on the peak of unreacted sulfate at the position expected for the reaction product APS.

Isolation and characterization of ^{35}S-labeled APS

The identity of the radioactive material in this shoulder was studied further as follows. During the elution of the 45-min sample (Fig. 4C), the radioactivity eluting between 7 and 8 min, the presumptive APS was collected, concentrated by rotary evaporation, resuspended in water and desalted with a G-25 column eluted with water. The APS eluting from this column was collected, again concentrated and analyzed by HPLC. Fig. 5 shows the HPLC profile obtained of the radiolabeled reaction product. A single radioactive peak was observed with a retention time on the chromatogram of that observed for APS. This finding was consistent with the conclusion that a product of the original reaction was APS.

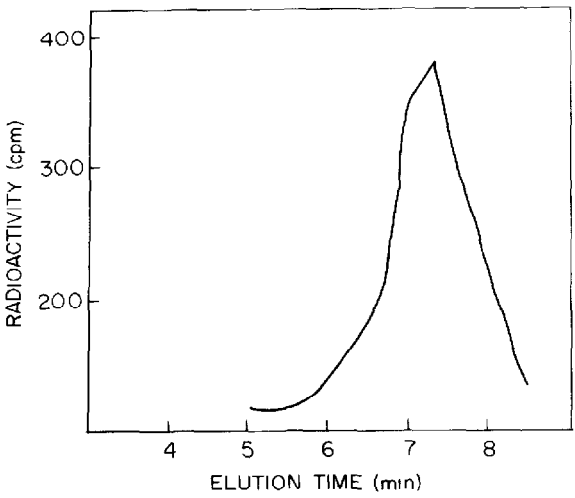

Fig. 5. Purification of [^{35}S]APS. The [^{35}S]APS formed in a reaction similar to the one described in Fig. 4 was purified as follows. The contents of the tubes containing the radioactivity eluting from 6.5 to 7.5 min (see Fig. 4) were pooled, concentrated and desalted on a G-25 column (15×0.5 cm). The column was eluted, fractions were collected and 2 ml of each analyzed for radioactivity by liquid scintillation counting. Those fractions containing radioactivity were concentrated by rotary evaporation, solubilized in mobile phase and analyzed by HPLC using conditions identical to those described in the legend to Fig. 2.

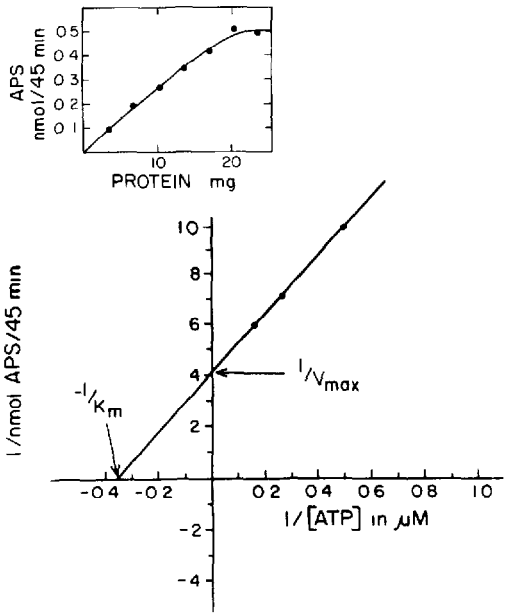

Fig. 6. Kinetic analysis of APS formation by the ATP-sulfurylase. Initial rates of APS formation were determined over a range of ATP concentrations and the results plotted in double reciprocal form. From these data, kinetic constants were determined. The inset shows the rate of APS formation as a function of a protein concentration in the extract.

Kinetics of rat liver ATP-sulfurylase activity

The activity of the ATP-sulfurylase from rat liver was characterized in more detail. The activity was studied as a function of protein concentration and as shown in Fig. 6 (inset), APS formation was proportional to protein through about 20 mg. To determine the kinetic constants, initial rates of APS formation were determined with ATP at concentrations between 2 and 5 μM. These data were plotted in double reciprocal form (see Fig. 6) and from these data an apparent Michaelis constant (K_M) of 3 μM and a maximal velocity (V_{max}) of 0.56 pmol/min per mg protein were calculated. Since some residual ATPase activity remains, these values are subject to some error.

DISCUSSION

The present study describes the development and application of a method to study an enzymatic activity after only a minimal amount of purification of the enzyme. To test the method, we have examined the ATP-sulfurylase activity in the tongue and liver from the adult rat. These tissues were particularly well suited for this purpose because both have significant levels of this activity as well as other activities such as APS-sulfohydrolase and APS-kinase, two activities that were expected to cause problems when measuring the ATP-sulfurylase in crude extracts. Thus, with these tissues, we could test the HPLC method for its capacity to measure the activity of the ATP-sulfurylase after only a minimum of purification.

As expected, the results of our initial studies on the ATP-sulfurylase revealed the formation of low levels of APS during the course of the incubation. But because we were able to measure the levels of ADP and AMP at the same time with this assay, we were able to determine that a significant amount of one of the substrates, ATP, was dephosphorylated by an ATPase and further that some of the APS was being degraded by APS-sulfohydrolase to AMP. Clearly, the activity of both of these reactions was affecting the yield of APS and, since it was possible to measure ATP, ADP and APS simultaneously, it was possible to determine easily the best condition to minimize the effects of these contaminating activities. Also, since the activity of these contaminating enzymes could be determined at the same time as the ATP-sulfurylase, it was a simple matter to determine that the level of ATPase and APS-sulfohydrolase was greater in the extracts from tongue than in liver.

The results we obtained on the ATP-sulfurylase activity with the HPLC method confirm those obtained with other methods. In agreement with other studies, we found this activity from rat liver to be soluble and obtained a pH optimum of 8.0. These values are similar to those previously reported for this activity (19, 20). The apparent K_M value that was obtained previously for the rat enzyme was 1.6 mM [21]. However, a significantly lower value of 0.38 mM was obtained from another source, *Penicillium* [22]. Our value was lower still, 3 μM, suggesting that the present HPLC method made it possible to optimize the conditions for the formation of APS by minimizing those reactions accounting for the loss of APS.

In conclusion the results of the present study have shown that HPLC can be

used to assay the activity of ATP-sulfurylase in preparations containing other activities such as ATPase, APS-kinase and APS-sulfohydrolase. The ability to measure the levels of the products of several reactions makes it possible to optimize reaction conditions for any one reaction without setting up new assays. The procedure is ideally suited to the assay of activities in situations where tissue is in limited supply.

ACKNOWLEDGEMENT

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